PHYSIOLOGICAL REVIEWS

VOL. 21 JANUARY, 1941 No. 1

THE NUTRITION OF INVERTEBRATES

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A science of nutrition, as McCay (125, 127) has emphasized, should rest on a knowledge of the nutritional needs of many different types of organisms. The nutrition of bacteria (104) and of vertebrates has been extensively investigated. Among the invertebrates, only the protozoa and insects have been studied to any extent with respect to their nutritional requirements. This review will summarize the considerable information which has accumulated since the subjects of protozoan and insect nutrition were last completely reviewed. It will also bring together what little is known concerning the nutritional requirements of other invertebrates.

By nutritional requirements is meant, not necessarily the natural food of the organism, but the fundamental chemical substances which the organism must get preformed from its environment in order to live, grow, and reproduce. For example, garbage may serve as a suitable food for rats, but it is not one of their nutritional requirements, in the sense in which riboflavin and certain amino-acids are requirements. One need only refer to the *Compendium of Culture Methods* (55) to see at once that a great deal more is known about the food of invertebrates than about their nutritional requirements. A study of the latter frequently involves special methods, such as rearing the organism in absolutely pure culture free from all other living organisms, including microscopic forms.

The invertebrates, like all other organisms, must somehow obtain energy, carbon, nitrogen, sulfur, phosphorus, and the mineral elements essential for the functioning of protoplasm. All the metazoa and protozoa thus far studied, other than certain of the free-living colorless flagellates, appear to require both their nitrogen and carbon already combined in organic compounds. They do not all require protein,

fats, and carbohydrates, and frequently proteins alone or their split products can serve as the nitrogen and carbon source. Recent work indicates that probably all of the metazoa and most of the protozoa require in addition accessory growth factors (i.e., substances which do not act as major sources of energy or building material but which are essential to the normal life of the organism, and which the organism is incapable of synthesizing) (cf. 104). Any detailed study of the nitrogen and carbon sources of an organism, like that of Rose (158) with rats, must be preceded by at least a partial purification of the accessory growth factors. Hence it is not surprising that much of the work to be reviewed has been concerned with the requirements for accessory factors, rather than with, for example, the protein or carbohydrate requirements.

I. THE PROTOZOA. *A. Free-living plant-like green and colorless flagellates.* This group of organisms, with affinities for the algae on the one hand and for the true protozoa on the other, shows all the possible gradations in complexity of nutritional requirements, from forms capable of autotrophic nutrition in light to forms requiring special growth factors. Except for the possession of pigment, the colorless flagellates of this group have their exact morphological counterparts among the chlorophyll-bearing species. From a physiological standpoint, however, it is very convenient to create a special group for the colorless forms. Lwoff (113, 115) designated them as leucophytes—protists having a plast but no chlorophyll and synthesizing starch or paramylum as their carbohydrate reserves,—and the green species as chlorophytes.

Since most of the green flagellates which have been studied have been found to be capable of autotrophic nutrition in light, in the same way as the green plants, the present discussion will be confined to results with the leucophytes and with chlorophytes grown in the dark.

1. *Carbon nutrition.* Pringsheim (152) first showed the greatly stimulating effect of acetate on pure cultures of *Polytoma uvella.* This effect of acetic acid and certain related compounds has since been demonstrated for all species of leucophytes and chlorophytes grown in the dark which have been investigated (115). The degree of stimulation varies greatly with the species concerned and the environmental conditions. For some species it is so great that growth in a peptone medium devoid of acetate is almost nil.

The effects of different organic acids have been observed by Lwoff and collaborators, Pringsheim, Loefer, and others, and have been re-

cently summarized by Provasoli (153), whose own work has contributed much valuable information. As is evident from table 1, acetic acid is the only organic acid which stimulated the growth of all species. For some species, however, acids up to and including myristic were active. If a species was able to utilize an alcohol, growth was about the same

TABLE 1

The growth response of various plant-like flagellates to organic acids and alcohols. (*From Provasoli, 1938*)

	CHLOROGONIUM EUCHLORUM	HYALOGONIUM KLEBSII	POLYTOMA OBTUSUM	CHLOROGONIUM ELONGATUM	CHLAMYDOMONAS AGLOEFORMIS	HAEMATOCOCCUS PLUVIALIS	POLYTOMA CAUDATUM	POLYTOMA UVELLA	ASTASIA QUARTANA	POLYTOMA OCELLATUM	POLYTOMELLA COECA	CHILOMONAS PARAMOECIUM	EUGLENA GRACILIS	EUGLENA GRACILIS VAR. UROPHORA	ASTASIA CHATTONI
Acid, acetic	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid, propionic	−	−	−	−	−	−	−	−	+	+	+	−	+	+	+
Acid, butyric	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid, isobutyric	−	−	−	−	−	−	−	−	±	+	−	−	−	+	±
Acid, valeric	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
Acid, isovaleric			−				−	−	±	±	−	±	−	−	−
Acid, caproic	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
Acid, isocaproic			−				−	+	+	+	±	+	±	±	±
Acid, heptylic			−				−	−	+	+	+	−	−	+	+
Acid, octylic			−				−	±		+	+	+	+	+	+
Acid, nonylic			−				−	−		+	+	±	+	±	+
Acid, decylic			−				−	−		+	+		+	±	±
Acid, lauric										+	±		+	±	±
Acid, myristic										±	−	±	±	±	±
Alcohol, methyl			−				−	−	−	−	−	−	−	−	−
Alcohol, ethyl	−	−	−				−	−	−	+	+	+	+	+	+
Alcohol, propyl			−				−	−	−	+	+	±	+	+	+
Alcohol, isopropyl							−	−	−	−	−	−	−	−	−
Alcohol, butyl			−				−	−	−	+	+	+	+	+	+
Alcohol, isobutyl			−				−	−	−	+	+	−	−	+	±
Alcohol, amyl			−				−	−	−	±	−	±	−	−	−
Alcohol, esilic										+	+	+	+	+	+

as with the corresponding acid, indicating a preliminary oxidation of the alcohol to the acid. Provasoli was able to demonstrate valeric acid in the fluid from a culture of *Polytoma ocellatum* grown in the presence of isoamyl alcohol. He suggested that the different species differ in their oxidative powers. In this connection Provasoli reported that

certain strains of *Polytoma obtusum*, which had been kept over a period of years on a peptone medium and had lost their ability to utilize butyric acid, recovered this ability after a prolonged period of adaptation. Flagellates thus adapted to butyrate could be adapted in a similar manner to caproate. These results suggest the formation of adaptive enzymes, instances of which have been previously reported for bacteria (104) but not for protozoa.

2. *Requirements for sulfur, nitrogen and growth factors.* Sulfur utilization has been studied only in the colorless flagellate *Chilomonas paramecium* (136). The fission rate was highest in media containing reduced sulfur (Na_2S, glutathione, cysteine, cystine), lower in media with sulfite and lowest in sulfate media. One of the chief functions of the sulfur appears to be in the oxidation of fat.

The nitrogen requirements of these flagellates have been elucidated in considerable detail. In the presence of light, almost all of the chlorophyll-bearing forms can utilize either the nitrate or ammonium radicals. The apparent exceptions, of considerable physiological interest, are all in the group Euglenidae (43). *Euglena gracilis* could use ammonium but not nitrate as its nitrogen source in completely inorganic media. *E. stellata* could use either ammonium or nitrate providing calcium was present. *E. deses*, however, could use neither ammonium nor nitrate but only various amino acids as the nitrogen source. *E. pisciformis*, with apparently still more complex nitrogen requirements, had to be supplied with peptone. Neither of the two latter species could be grown in the dark. It should be noted, however, that a requirement for peptone may indicate the need for a specific growth factor rather than for a complex source of the bulk of the nitrogen (see below). Moreover, it may be possible to grow *E. pisciformis* in an inorganic medium of different composition from any thus far tried. Thus, although Hutner (102) concluded that *E. anabaena* var. *minor* required a complex nitrogen source, Hall (72) was able, by suitably modifying other constituents of the medium, to grow it autotrophically with ammonium nitrate as the sole nitrogen source. Similarly, *E. gracilis* could be readily grown under autotrophic conditions, but only if manganese was present in the medium (76). This subject has been recently reviewed by Hall (73).

Among the colorless flagellates and the green flagellates grown in the dark, some, like *Polytoma uvella*, can use ammonium as the sole nitrogen source. It is interesting to note that *P. uvella* can do so only if a ferric salt is added to the medium. Other species apparently require amino acids or complex peptones. The recent work of Lwoff and Dusi (118)

indicates that peptone may act by furnishing growth factors, notably the pyrimidine and thiazol of thiamin. This discovery has made it possible to grow a number of these flagellates in entirely synthetic media containing ammonium acetate, iron, suitable salts and either the pyrimidine or thiazol of thiamin or both together (116).

Mast and Pace (135) reported that they could grow *Chilomonas paramecium* not only in simple organic media, such as those which support growth of *Polytoma uvella*, but also in a completely inorganic medium. In this medium, ammonium served as the nitrogen source and carbon dioxide (in a ratio of 1 part CO_2 to 5 parts of air) as the carbon source. In the inorganic medium, silicate, which stimulated growth in the simple organic medium, was essential for growth. The cultures grew autotrophically in the dark as well as in the light and there was slight evidence for the formation of nitrate. On this basis it was assumed that the energy necessary for the reduction and utilization of carbon dioxide was derived from the oxidation of ammonium to nitrate, as is the case with certain autotrophic bacteria.

The work of Mast and Pace has not been confirmed by other investigators (111, 75). Lwoff and Dusi's (118) recent very careful work seems to show conclusively that *Chilomonas* can grow with ammonium as its nitrogen source and acetate as its carbon source only if both the pyrimidine and thiazol of thiamin are supplied. These results do not eliminate the possibility that *Chilomonas*, if provided with a high carbon dioxide concentration, silicate, and the essential amounts of pyrimidine and thiazol, could obtain the bulk of its carbon requirements from the carbon dioxide. Burrows (19) found that *Chilomonas* could be induced to grow, although with difficulty, in both the simple organic medium and the inorganic medium with high carbon dioxide tension of Mast and Pace. Growth was light, although it apparently could be carried on indefinitely. In spite of the use of very sensitive tests for nitrite and nitrate neither of these substances could be detected in cultures in the inorganic medium. If the flagellates were actually using carbon dioxide as their carbon source, the manner in which they obtained energy for its reduction remains a mystery. Burrows tested his cultures very thoroughly for bacteria, including nitrifying forms, with uniformly negative results. A source of error was his utilization of cotton plugs, which also cannot be used in careful studies with autotrophic bacteria (184). Then, too, it is possible that under his conditions sufficient numbers of flagellates died in each culture to liberate enough organic substance for another light culture.

Elliott (46) found no effect of "pantothenic acid" on the growth of the

chlorophyte *Haematococcus pluvialis*. He did obtain a marked effect with certain of the so-called plant hormones on the growth in light of *Euglena gracilis* (49). Indole-acetic acid accelerated growth at a concentration of 1:10,000,000, as did indole propionic acid, while indole butyric was active down to a concentration of 1:1,000,000. The effects were much greater at pH 5.6 than at pH 7.4. Interestingly enough, corresponding dilutions of these substances had either no effect or an inhibitory effect on the colorless euglenoid *Khawkinea halli*.

B. Free-living amoebae and ciliates. Comparatively little work has been done with amoebae and ciliates in absolutely pure culture, a condition necessary to any study of their nutritional needs. Oehler (146, 147) was the pioneer in this field and obtained one species of ciliate and several species of amoebae in pure culture. Glaser and Coria (61) grew *Trichoda pura* in their sterile hay infusion-serum medium. Since then a number of others of the smaller species of ciliates have been reared free from contaminants (see Hetherington, in 55).

The one species of ciliate which could be most easily grown under bacteria-free conditions and which has lent itself best to studies on nutrition is *Glaucoma pyriformis* (synonyms of which are, according to Hetherington, *Saprophilus oviformis* and *Colpidium striatum*). One strain of this ciliate utilized, as judged by acid production (34), levulose, glucose, galactose and maltose, but not mannite, arabinose, xylose, sucrose, lactose, inulin, or starch while another strain (*Colpidium striatum* (47)) utilized starch, levulose, mannose, and dextrose but not galactose, dextrin, inulin, salicin, melezitose, sucrose, lactose, maltose, or mannite. The closely related ciliate *Colpidium campylum* (47) fermented the same carbohydrates as the latter strain of *Glaucoma* and also maltose. The dextrose consumption of *C. campylum* and *Glaucoma pyriformis* cultured in a tryptone salt solution medium has been found to be 2.34 to 3.92 $\times$ 10^{-7} mgm. per organism per hour for *C. campylum* and 0.5 to 3.29 $\times$ 10^{-7} per organism per hour for *G. pyriformis* (112). Glaser and Coria (63) found that *Saprophilus oviformis* (a hot springs strain of *Glaucoma pyriformis*) and *Trichoda pura* fermented glucose and maltose but not lactose or sucrose. If a suspension of cellulose was added to cultures of these two species of ciliates, or of *Chilodon cucullus*, *Paramecium caudatum*, or *P. multimicronucleatum*, the turbidity was soon cleared away, indicating the digestion of cellulose by the protozoa.

Elliott (48), working with *Glaucoma pyriformis* (*C. striatum*) and *Colpidium campylum*, confirmed Lwoff's (113) conclusion that these

ciliates could not grow with single amino acids or even with mixtures of several amino acids, but required substances present in peptones. These two species could not grow continuously in media consisting of zein, gliadin, gelatin, or silk peptone, although they did grow in meat peptone (74). Supplementing gliadin with lysine, or gelatin with tryptophane and isoleucine, had no effect. However, the addition of 0.00005 per cent Difco yeast extract permitted continuous growth in gelatin, zein, or gliadin, suggesting a requirement for special growth factors (see below). In the presence of such a small amount of yeast extract, growth in a gliadin medium was considerably enhanced by the addition of lysine.

The culture by Glaser and Coria (62) of *Paramecium caudatum* free from living microörganisms first emphasized the very complex nutritional requirements of certain free-living protozoa. Numerous investigators had grown *Paramecium* in the presence of pure cultures of live bacteria or yeast. But if the ciliates were washed free from the living microörganisms and furnished with corresponding dead microörganisms, no growth occurred. However, if heat-killed yeast was supplemented with a suitable liver extract and a fragment of rabbit kidney aseptically removed from the animal, excellent growth ensued under completely sterile conditions. All three constituents of the medium were essential for continuous cultivation, but the kidney tissue could be replaced by an extract of kidney sterilized by Berkefeld filtration. It is apparent that *Paramecium* requires numerous special growth factors which, in nature, are supplied to it by the living microörganisms always present in its habitat. The chemical nature of these factors has not as yet been investigated.

"Pantothenic acid," a partially purified yeast growth factor, stimulated the growth of *Glaucoma pyriformis* (*Colpidium striatum*) in the pH range of 5.5 to 6.5, while at pH's above 7.0 growth was slightly less with the acid than without it (46). Pantothenic acid used in place of yeast extract in a zein, gliadin, or gelatin medium did not support growth. Hence it is not the essential substance which is lacking in these proteins and in hydrolyzed silk. Lwoff and Lwoff (119) have shown that this essential substance is thiamin. The addition of thiamin to a hydrolyzed silk-glucose medium permitted continuous growth of *G. pyriformis*. There was no growth if thiamin was replaced by either its pyrimidine or its thiazol. Evidently, *Glaucoma* resembles mammals in requiring the intact thiamin molecule. Thiamin could not be replaced by a number of closely related compounds (120).

Elliott (199) has more recently confirmed this result with his strain designated as *Colpidium striatum*. Thiamin, when added to a tryptone medium rendered itself unsuitable for growth by an hour's autoclaving at pH 9.6, permitted optimum growth at concentrations down to 1:10,000,000. Crystalline riboflavin and a vitamin B_6 concentrate had no effect on the growth of the ciliates. In media not containing dextrose, pimelic acid at a concentration of 10^{-6} grams per ml. stimulated the growth of *Colpidium campylum* (200), but it had no effect in a gelatin dextrose medium in which the growth is always much greater than in dextrose-free media. No evidence was obtained to indicate that pimelic acid was essential for growth. A valuable study by Kidder, Lilly and Claff (201) shows the tremendous effects which type of food may have on the morphology of a protozoon. *Glaucoma vorax* reared on pure cultures of live bacteria had a characteristic tailed shape, which it lost when reared in a sterile peptone-broth medium. When allowed to feed on the related ciliate *Colpidium campylum*, the *G. vorax* increased many times in size, an effect which was not produced by yeast or flagellates, or by dead *Colpidium* or various extracts of them.

Among the free-living amoebae, *Acanthamoeba castellanii* (113) and *Mayorella palestinensis* (154) have been grown free from other organisms. Reich cultured *M. palestinensis*, a soil amoeba, in a medium consisting of a balanced salt solution containing 1 per cent peptone and 1 per cent dextrose. The peptone could be replaced by serum, gelatin, or autolyzed yeast, but not by single amino acids or ammonium salt. Dextrose was indispensable for good growth, although no acid was produced. Levulose, sucrose, and lactose could replace dextrose.

Acanthamoeba castellanii, which grows well in meat peptone but not in silk peptone or in meat peptone heated 20 minutes at 120°C. at pH 9.6, grew in the two latter media if either thiamin or its pyrimidine was added (117). The ciliate *Glaucoma pyriformis*, which requires the intact thiamin molecule, developed well in a peptone medium devoid of thiamin but containing its pyrimidine, its thiazol, and a rich culture of *Acanthamoeba*. It is evident that the amoeba can synthesize thiamin from its pyrimidine and its thiazol.

C. The parasitic protozoa. 1. *Hemoflagellates.* A thorough understanding of the nutritional requirements of parasitic protozoa can be obtained only if the organisms are grown in pure culture. This fact explains why more progress has been made with the hemoflagellates than with any other group. Most of these organisms, which occur

principally in the blood of vertebrates or in the alimentary tract of insects, can be readily cultured in media containing blood. The species from vertebrates are already in a practically sterile environment while those from insects can be rather easily freed from bacteria.

The carbohydrate metabolism of the hemoflagellates has attracted considerable attention, especially since Yorke, Adams and Murgatroyd (196) first showed that the pathogenic trypanosomes, except *Trypanosoma cruzi*, require glucose and utilize as much as 7 to 8 mgm. per 1,000 million organisms per hour at 37°C. Since the subject of the metabolism of the pathogenic trypanosomes and its relation to the carbohydrate metabolism of their hosts has been recently reviewed by von Brand (17) it will not be further discussed here.

The other aspect of the nutrition of hemoflagellates which is of great interest is the apparent requirement of many of the species for blood. Cleveland and Collier (31) found that 7 species of *Leptomonas* could be grown in simple glucose-peptone media, while several species of *Leishmania* and *Trypanosoma* gave good growth only in the presence of hemoglobin or hydrolyzed hemoglobin. Lwoff (114) showed that *Strigomonas* (*Leptomonas*) *fasciculata* could grow in peptone solution only in the presence of blood, and that under proper conditions and at blood concentrations of 1:200,000 to 1:500,000 the growth of the flagellates was proportional to the concentration of blood. Blood greatly stimulated the respiration of these protozoa. Lwoff (116) has recently reviewed the literature on the rôle of hematin in the growth of certain hemoflagellates and the hemophilic bacteria. The iron content and the peroxidase activity of hematin are not responsible for its effect, since protoporphyrin, having no iron and no peroxidase activity, affects the respiration and growth of *Strigomonas fasciculatus* just as does hematin, whereas hematohemin, which has both iron and peroxidase activity, has no effect on the flagellate. It seems probable that the hematin or protoporphyrin is used for the synthesis of essential respiratory enzymes. In this connection, it is most interesting that the bacterium *Hemophilus influenzae* does not require hematin if grown anaerobically.

Hematin is not the only growth factor required by the hemoflagellates. *Strigomonas oncopelti*, which does not require hematin and grows in meat peptone but not in hydrolyzed silk, requires the complete thiamin molecule. If this is added, at concentrations as low as 1×10^{-9}, to a hydrolyzed silk-glucose medium, good and continued growth results (121, 122). Thiamin cannot be replaced by its pyrimidine or its thiazol added either separately or together, or by certain related com-

pounds. *Strigomonas fasciculata* and *S. culicidarum* similarly require thiamin, as well as hemin.

Schizotrypanum (*Trypanosoma*) *cruzi*, the most readily cultivated of the pathogenic trypanosomes, has been shown (123) to require ascorbic acid as well as hematin and other as yet unidentified growth factors. This flagellate would not grow in peptone-saline unless a rather large amount of blood was added. It did not grow in peptone with inactivated serum or in peptone with washed or laked blood cells, but did grow in peptone plus serum plus washed blood cells. Peptone with serum and hematin gave no growth. If synthetic l-ascorbic acid was added to the peptone-serum-hemin medium, good growth ensued. All these materials were shown to be essential for growth. *Leishmania tropica* is stated to have the same nutritional requirements as *S. cruzi*.

2. *Intestinal and related flagellates.* Chatton (23) first obtained bacteria-free cultures of an intestinal flagellate, *Trichomastix colubrorum*, in a medium containing blood or a piece of an organ. Cleveland (29) grew the coprophagous organism, *Tritrichomonas fecalis*, in media containing heat-killed bacteria. Witte (185) and Glaser and Coria (64) independently and by different methods obtained pure cultures of *Trichomonas foetus*, the former in a peptone-bouillon-blood medium, the latter in the Locke-egg-blood medium of Kofoid and Wagener (107). At the same time Bos (16) first obtained pure cultures of *Trichomonas columbae*, a parasite of pigeons which invades certain organs of its host. This flagellate could be grown in Locke-egg-serum or in bouillon with liver fragments (autoclaved).

Riedmüller (156) concluded that the growth factors required by *T. foetus* were present in blood serum rather than in the blood cells. Growth was greatly stimulated by glucose, galactose, fructose, lactose, maltose, sucrose, raffinose, dextrin, starch, and glycogen, and with all these carbohydrates a decrease in pH occurred. With pentoses or higher alcohols there was neither growth stimulation nor a decrease in pH. Andrews and von Brand (3) performed quantitative studies on glucose consumption by *T. foetus*.

Cailleau (20) investigated in exhaustive fashion the nutritional requirements of *Eutrichomastix* (*Trichomastix*) *colubrorum*, *Trichomonas columbae* and *T. foetus*. *Eutrichomastix colubrorum* did not grow if whole blood was replaced by rabbit serum, but did grow if the serums of various other species of animals were used, guinea pig and horse serum being best. This flagellate produced much acid with glucose, galactose, levulose, maltose, sucrose, and raffinose, little with lactose, and none

with arabinose, xylose, rhamnose, inulin, dextrin, glycerin, erythrite, sorbite, dulcite, or mannose. *Trichomonas columbae* could be grown in an autoclaved medium containing fragments of veal liver in bouillon in a ratio of about 1:6. In such a medium, a strong acidity was developed with glucose, galactose, maltose, sucrose, dextrin, and soluble starch, little with levulose, lactose, and inulin, and none with arabinose, xylose, rhamnose, raffinose, glycerin, erythrite, sorbite, mannite, or dulcite. Pigeon, rat, or guinea pig serum, but not horse, rabbit, human, or cat serum, supported growth if added to bouillon. Liver extracts prepared with water, alcohol, acetone, or ether could not replace whole liver. The residue left after acetone extraction could not do so, but this residue combined with the acetone extract could. In testing for the nature of the acetone soluble factor, the basic medium used was bouillon with liver which had been extracted with acetone and alcohol. It was soon found that the acetone extract could be replaced by pure cholesterol at dilutions as high as 1:10,000 (when added in alcoholic solution to give a fine suspension in the medium). Cailleau then tested 66 sterols and sterol derivatives as growth factors for *T. columbae* and found certain correlations between chemical structure and activity. For instance, shortening or suppressing the side chain of cholesterol eliminated the activity.

T. foetus and *Eutrichomastix colubrorum* also require cholesterol, or other suitable sterols (21, 22). Ascorbic acid is another growth factor required by these flagellates. Thus *E. colubrorum* grew in a medium containing bouillon, egg albumin, cholesterol, ascorbic acid, and peptone, but did not grow if any one of these constituents was omitted (22).

Some of the polymastigote and most of the hypermastigote flagellates of many species of termites and of the roach *Cryptocercus punctulatus* (32) are of special interest in that they apparently must have cellulose as their chief carbon source and soon die out if their host is fed on other carbohydrates. The ability of these flagellates to digest cellulose and in some way to provide food for their insect hosts was proved by the early work of Cleveland (26). Later work has only served to confirm and extend his conclusions (170, 171, 100, 101).

3. *Other intestinal protozoa.* Although, following the original work of Boeck and Drbohlav (14), a number of species of parasitic amoebae have been cultured, little is known concerning their nutritional requirements. One reason for this lack of knowledge is that they have not yet been grown free from bacteria. Cleveland and Sanders (33) obtained bacteria-free abscesses of *Entamoeba histolytica.* When the amoebae

from such abscesses were placed in various sterile culture media only slight multiplication occurred and the cultures died out within 14 days. The amoebae grew well if supplied with living pure cultures of various bacteria, but dead bacteria supported no growth. The work of Dobell and Laidlaw (41) and many others after them has shown that in the ordinary contaminated cultures of *E. histolytica* the addition of starch enhances growth and enables encystation to occur.

4. *The sporozoa.* Nothing is known concerning the nutritional requirements of this vast class of protozoa, almost all of which are intracellular parasites and none of which have ever been cultured *in vitro*. The so-called "cultures" of malarial parasites, including those most recently obtained by Hewitt (87), represent at best a short survival with the production of one asexual generation. Christophers and Fulton (24), using concentrated suspensions of monkey red blood cells 90 per cent of which contained large parasites of *Plasmodium knowlesi*, found that glucose was consumed, although its addition produced only a small increase in oxygen uptake.

II. Insects. The food of insects presents an almost infinite variety, but it may well be that the fundamental nutritional requirements of the different species are very uniform, but are satisfied in different ways and from different sources. Uvarov (177) pointed out the many unsolved problems connected with insect nutrition. Considerable progress, some of it summarized by Wigglesworth (182) and by Hoskins and Craig (97), has been made since then toward the solution of certain of these problems. In this review, emphasis will be laid on the more recent work which is leading to a knowledge of actual chemical substances required for the various activities of insects. The extensive literature concerned with digestive enzymes will be omitted, except as it indicates some special nutritional requirement.

A. Carbon and nitrogen sources. The carbohydrates and related compounds which insects are able to utilize have been thoroughly studied for a few species. Adult honey bees lived well on solutions of glucose, levulose, sucrose, maltose, and trehalose (151). They could also utilize melezitose and possibly mannose, but not raffinose, lactose, galactose, dextrin, starch, inulin, rhamnose, xylose, arabinose, mannite, amygdalin, or salicin. Vogel (179) suggested that the failure of the bees to survive on certain sugars in Phillips' experiments may have been due to the non-ingestion of these sugars, perhaps because they were tasteless. She repeated Phillips' work, using in all the solutions enough $\frac{M}{4}$ sucrose to provide each bee with 0.006 ml. per day. This amount of

sucrose did not prolong life over that of bees given water only. Of the sugars which bees could taste, glucose, fructose, α-methyl glucoside, maltose, trehalose, melezitose and raffinose could all be utilized. Of the tasteless sugars, arabinose, xylose, galactose, mannite, dulcite, sorbite, and cellobiose were utilized while rhamnose, mannose, sorbose, lactose, and melibiose were not. Bertholf (13) placed honey bee larvae in carbohydrate solutions and compared the length of life (a matter of hours) with that of controls in water. Sucrose prolonged life 7.5 times over the water controls; maltose, melezitose, dextrose, and honeys about 4 times; trehalose and dextrin 3 times; galactose and lactose 2 times; starch and glycogen not at all.

Adults of the fleshfly *Calliphora erythrocephala*, which live one to two months on sucrose solution as compared with two to three days on pure water, could use glucose, fructose, galactose, mannose, sucrose, maltose, trehalose, melibiose, lactose, raffinose, melezitose, xylose, α-methyl-d-glucoside, starch, glycogen, mannitol and sorbitol, but could not use sorbose, cellobiose, arabinose, rhamnose, β-methyl-d-glucoside, helicin, arbutin, salicin, inulin, erythritol, dulcitol, or inositol (52). All the substances which were utilized were as effective as cane sugar except xylose, lactose, starch, and glycogen, on which the flies lived only one or two weeks.

Sorbitol gave significantly longer life of *Calliphora* adults than any other carbohydrate (202). Glycol, dihydroxy-acetone, alanine, glycine, butyric acid, isobutyl alcohol, and lactic, pyruvic, succinic, malic, or citric acids did not permit survival of the flies, but on glycerol they were able to survive 6 to 8 days (202). Adults of the apple maggot survived about a month when fed dextrose, levulose, sucrose, maltose, galactose or honey, but only a few days when fed lactose or starch (203).

The larvae of *Tenebrio molitor* did not grow in the absence of carbohydrate (204). When added at a concentration of 20% to a basal diet of protein, salts, and yeast extract, starch, mannitol or levulose produced rapid growth of the mealworm larvae; glucose, sucrose, xylose, lactose or glycogen produced slow growth; while arabinose, galactose and inulin produced no growth.

The utilization of cellulose as a chief carbohydrate source by certain insects is a subject of special interest, since relatively few animals are able to digest this polysaccharide. Cleveland (27, 30) showed that termites, which feed almost exclusively on wood, are actually unable to use cellulose directly and must have this substance digested for them by certain of their intestinal flagellates. The same situation exists in the

wood-feeding roach, *Cryptocercus punctulatus* (32). Following the work of Cleveland, a notion arose that all insects which are able to utilize cellulose as a food can do so only by the grace of microörganisms of one sort or another living in symbiosis with them. Recent work shows definitely, however, that some insects which feed on wood do not utilize the cellulose but actually subsist on sugars, starch, etc., in the wood, while others, which have no symbionts, themselves produce a cellulase (157, 130, 131).

Almost nothing is known as to which fats, if any, must be present in the diet of insects. Becker (12) found that mealworms (*Tenebrio molitor*) got along very well on meal with its starch removed but did not eat defatted meal. Some species, such as the beet leafhopper (54), can synthesize fats from carbohydrates, and such species may not require dietary fat. *Blatella germanica* can synthesize fat from protein (137). In general, the nature of the fat deposited in the fat body of insects depends, as in mammals, on the nature of the fat in the diet. Such is the case, for example, in the beetle *Pachymerus dactris* (36) and in the larvae of *Lucilia sericata* (197).

The apparent utilization of wax by the caterpillars of *Galleria mellonella* provides another example of an unusual type of nutrient material. Larvae fed on the impurities of comb wax, or on albumin, cereal, or sugar with albumin, failed to grow, but they did grow very well if any of these nitrogenous foods was supplemented with pure wax (139). Wax could be replaced by either of its two main components, cerine and myricine, but not by palmitic or stearic acid. A mixture of a nitrogenous food with water permitted good growth and metamorphosis. Metalnikov concluded that the wax normally served in place of water in the perfectly dry comb. Dickman (40) confirmed Metalnikov's finding that much of the wax passing through the gut of a *Galleria* larva was assimilated. Duspiva (44) showed that *Galleria* larvae utilized the fatty acids, esters, and alcohols of wax, but not the hydrocarbons.

The meager evidence concerning the nitrogen needs of insects indicates that they require a full complement of amino acids. Pure silk fibroin did not suffice for the nutrition of larvae of *Anthrenus museorum* but the combination of fibroin with sericin did (1). Incomplete proteins were insufficient for cockroaches (124) and for fleshfly larvae (141). *Drosophila* larvae require complete proteins or hydrolysates of complete proteins (109). Mixtures of amino acids, including threonine, did not support growth, suggesting that the amino acid requirements of *Drosophila* may be more complex than those of rats. Alcoholic yeast extract was present in all the media as a source of growth factors.

The ability of a few types of insects to utilize keratin, a protein which is not attacked by proteinases under the usual conditions, has been recently elucidated (110, 45). Such insects (as clothes' moth larvae, *Mallophaga*), and only such insects, show in the gut a strong reducing condition which makes keratin susceptible to digestion by ordinary proteolytic enzymes (67).

B. Requirements for growth and differentiation. 1. *Orthoptera.* The findings of McCay (124, 128) and Melampy and Maynard (137) show that *Blatella germanica* requires, in addition to carbohydrate and a complete protein, heat stable materials present in alcoholic yeast extract. On a diet of casein, starch, cellulose, sugar, lard, and minerals the roaches could live as long as two months, but no growth whatever occurred until yeast or yeast extract was added. The alcoholic yeast extract could not be replaced by a rice polishings extract containing both vitamins B_1 and G. The roaches also required an ether-soluble factor present in wheat.

2. *Isoptera.* Cook and Scott (37) found, contrary to Cleveland's (28) earlier results with *Reticulitermes*, that the termite *Zootermopsis angusticollis* lost weight on a diet of mercerized cotton changed daily. If the cotton was not changed, the termites gained as much weight as on a natural diet of wood not changed. Various foods were incorporated in a 2 per cent agar gel, itself of no nutritional value. The food and containers were changed daily. The termites gained weight on a diet of salts, casein, sucrose, or glucose, cod liver oil, and rice polishings extract. The omission of any constituent of the diet resulted in loss in weight.

3. *Lepidoptera.* Larvae of the Mediterranean flour moth, *Ephestia kuehniella*, required a fat soluble substance present in whole wheat, egg yolk, and butter (155). The larvae grew very slowly on a highly milled flour and the moths which emerged weighed only 50 to 60 per cent as much as those reared in whole wheat flour. Supplementing the highly milled flour with olive oil, lard, butter, ether extract of egg yolk, casein, or a salt mixture did not improve growth, but the addition of 1.4 per cent solids from a 50 per cent alcoholic extract of bakers' yeast did.

The growth of clothes' moth larvae (*Tineola bisselliella*) is greatly accelerated by yeast (35). Whereas a lot of 100 larvae on raw wool alone gained only 3 per cent in weight after 4 weeks, another lot fed on raw wool with yeast gained 32 per cent. The larvae grew normally on a diet of vitamins A and B free casein plus vitamins A and B free lactalbumin plus Harris yeast extract (38). *Galleria mellonella* larvae grew well on pollen or on various complete food mixtures incorporated

in honey and glycerin. Wax was not required. Growth was best in mixtures containing yeast, but second generation larvae reared on yeast with sawdust only were unable to metamorphose successfully and died in the late pupal stage (84).

The larvae of *Galleria mellonella*, which grew well on a diet of 1 part autoclaved yeast and 2 parts wax, did not grow if the autoclaved yeast was thoroughly extracted with water and alcohol (205). Such a deficient diet could be made suitable for normal growth and development by supplementing it with 5 to 10 γ of nicotinic acid per 100 grams of medium.

Other positive results obtained with the feeding of known growth promoting substances to lepidopterous larvae are those of Grandori, Provasoli, and Giorgi (69). Certain races of silkworms, when fed or injected with tyrosine or lactoflavin, or a mixture of these two substances, spun heavier cocoons than the untreated controls. The effect of tyrosine was especially noticeable in groups reared on greenhouse leaves in April.

4. *Coleoptera.* Sweetman and Palmer (166) showed that *Tribolium confusum* did not grow on commercial wheat embryo extracted with alcohol and ether, but did grow if the alcoholic fraction alone was returned to the residue. The beetles grew normally on a diet of casein, dextrin, and salts only if it was supplemented with an alcoholic extract of wheat germ. The *T. confusum* larvae require at least two accessory factors, one contained in rice polishings extract, the other in autoclaved yeast (164). If the latter was present at a level of 1 per cent in the diet, the length of the larval stage was, within certain limits, inversely proportional to the amount of rice polishings extract supplied. *Tribolium* also requires phosphorus but not vitamin D (142). The other common flour beetle *Tenebrio molitor* does not grow and metamorphose on purified wheat flour or polished rice unless these are supplemented with a small percentage of the grain husks, or with egg white (149).

Two thorough studies of the nutritional requirements of larvae of *Tribolium confusum* have recently appeared (206, 207). In one (206), the basal diet contained casein and had to be supplemented in order for growth to occur with thiamin, riboflavin, an alkali-treated yeast extract (B_x), ergosterol and a water and ether insoluble material (factor U) present in yeast, liver and egg yolk. The optimal amounts of the known growth factors in 1.5 grams of diet were 6 γ of thiamin and of riboflavin, and 100 γ of ergosterol. The fat body of larvae kept on a diet deficient in thiamin or B_x consisted of small cells poor in fat. In

the other study (207), the basal diet contained egg white and had to be supplemented with thiamin, riboflavin, alkali-treated yeast extract (B_x), ergosterol and histidine. It is concluded that in this diet Factor U was supplied in the egg white, whereas in the other diet histidine was supplied by the casein. The factor B_x could not be replaced by bios I, II, or III, or combinations of them. *Oryzaephilus surinamensis*, whether with or without symbionts, had the same nutritional requirements as *Tribolium*, except that it appeared not to require Factor U (206).

Payne (150) has shown the need of two species of coleopterous larvae, *Synchroa punctata* and *Dendroides canadensis*, for special pupation inducing substances in their food. She placed some grown larvae of each species in vials with sterilized oak bark, while others were placed in vials with unsterilized oak bark or with mycelium of *Armillaria nigra*, a fungus commonly found in dead and dying trees. All of the two latter groups pupated and emerged as adults within a few months. Of the larvae kept on sterilized bark, none pupated, although many of them survived as apparently healthy larvae for 6 years. Every half year during this time a few of the larvae of this group were removed and fed on unsterilized oak or rhizomes of the fungus *A. nigra*. Such larvae pupated within 5 days, a dramatic effect of the essential pupation-inducing substances which must be present in *A. nigra* and possibly other microörganisms.

Koch (105) found that the yeast-like symbionts of *Sitodrepa panicea*, present in the cells of special pouches at the anterior end of the midgut, furnish their host with essential growth factors. When symbiont-free larvae were placed on a diet of condensed pea soup, on which normal symbiont-containing larvae grow very well, they failed to grow. A corresponding group of symbiont-free larvae fed the pea soup with 25 per cent of irradiated dried yeast grew normally. The symbionts evidently enable the host to live on a diet deficient in certain growth factors which are present in yeast. There are at least two heat stable factors concerned (106).

Larvae of the *Lyctus* powder-post beetle do not utilize the skeletal substance of wood (148). They required for normal development a substance which could be extracted from oak sapwood with water at a temperature of 60°C. Extraction of the wood with fat solvents did not alter its suitability as food. The beetles were reared on wood-free diets containing starch, sugar, and protein.

The hide beetle, *Dermestes volpinus*, grew almost as well on a diet of

casein, cystine, cholesterol, salts, and 10 per cent yeast as on a control fish meal diet (56). On a diet of yeast and salts only, the larvae grew well at first but rapidly died off at the third molt. With yeast, salts, and cholesterol, growth was even better than on the fish meal diet. *D. volpinus* is the third species of insect shown to require cholesterol (see *Diptera* below).

5. *Diptera.* Delcourt and Guyénot (39) early recognized the importance of a strictly controlled environment for the proper interpretation of experiments concerned with inheritance in *Drosophila.* They succeeded in rearing the fruit flies under aseptic conditions with autoclaved yeast suspension soaked up on cotton as the sole food. Guyénot (71) showed that under sterile conditions *Drosophila* larvae require, in addition to peptone and salts, lecithin (or some impurity in it) and an alcohol-soluble fraction of yeast autolysate. Bacot and Harden (7) essentially confirmed these results. On a diet of purified caseinogen, starch, salts, and cane sugar, both butter fat and yeast extract were necessary for growth to occur. Yeast extract could be replaced by wheat germ extract. Butter fat appeared to supply some fat soluble material other than vitamin A.

Two of the growth factors supplied by yeast extract have been shown to be vitamin B_1 and lactoflavin (95, 96). However, these two pure substances together could not replace yeast extract in a diet consisting of a peptic digest of casein, sucrose, the unsaponifiable fraction of arachis oil, salts, and yeast extract. The unsaponifiable fraction of fat could be replaced by pure cholesterol or ergosterol or by larger quantities of sitosterol, stigmasterol, or phytosterol, but not by calciferol or lumisterol.

Tatum (168) has shown that *Drosophila* larvae require under sterile conditions three fractions of yeast in addition to their carbohydrate, amino acid, cholesterol, and known vitamin requirements. In the presence of any one of the three factors no growth occurs. Factors II and III together enable growth and development to occur but at a slower rate than in the presence of all three factors. If factor I and either II or III are present larval growth is rapid but the insects die as pupae. Tatum has also confirmed the need of *Drosophila* larvae for riboflavin and has demonstrated that they require nicotinic acid, 10γ per 10 ml. of medium being the minimum amount which had to be added to a medium of 1 per cent alcohol-extracted dry yeast, 1.5 per cent agar, and 1 per cent sucrose in order to permit normal growth.

Beadle, Tatum and Clancy (11) and Khouvine, Ephrussi and Chevais

(103) have recently found that partial starvation of *Drosophila* larvae induces the formation of the so-called v^+ hormone (responsible for eye pigmentation) in vermilion larvae which ordinarily lack it. The v^+ hormone is also synthesized from tryptophane by an unidentified bacillus, which, when ingested by vermilion larvae, modifies the eye color of the resulting adults (169).

Housefly larvae, like *Drosophila* larvae, feed in nature on media rich in microörganisms. Glaser (60) succeeded in growing *Musca domestica* under sterile conditions through several generations by using as a medium for the larvae a mash made from heated whole minced swine liver coagulum, dried brewers' yeast and fine pine sawdust.

The fleshfly *Lucilia sericata* has been extensively studied from the nutritional standpoint. Wollman (187) grew the larvae under sterile conditions on sterilized sheep's brain. Michelbacher, Hoskins and Herms (141) used a sterile synthetic diet consisting of highly purified casein, yeast, salts, butter or cod liver oil, and cystine. Growth was as good as on contaminated meat or fish. If the cystine was omitted, growth was good but the pupae were irregular in shape. Hobson (91) reared *Lucilia* larvae on an autoclaved diet of liver and yeast incorporated in nutrient agar. A factor present in yeast was shown to be essential if the larvae were kept under sterile conditions. Yeast autolysate contained at least two heat stable factors and one heat labile one. The latter was shown to be vitamin B_1 (92). Hobson (93, 94), working with *Lucilia*, demonstrated for the first time the requirement of an organism for cholesterol in its food. The basal diet consisted of a 15 per cent meat peptone solution, thoroughly extracted with ether to remove the last traces of fat, and a small amount of aqueous yeast extract. Under these conditions cholesterol was the only fat soluble substance required for growth. Other sterols were less active than cholesterol.

The nutrition of mosquito larvae has also attracted considerable attention. These insects, in common with the other dipterous larvae already discussed, ingest under natural conditions large quantities of microörganisms of all kinds. Barber (9, 10) was able to rear them on pure cultures of bacteria and protozoa, but all attempts to rear them in the absence of living microörganisms proved fruitless. Hinman (89) obtained the development of small numbers of bacteria-free *Aëdes aegypti* mosquitoes in water rich in organic matter and sterilized by filtration through a Berkefeld N candle. He later (90) attempted unsuccessfully to rear *A. aegypti* in various autoclaved media, on

bacteria killed by heating at 60°C. or by means of formaldehyde, and in filtrates from bacterial cultures. Trager (172) found that *Aëdes aegypti* larvae could be readily grown under sterile conditions in a medium consisting of an autoclaved 0.5 per cent solution of Lilly liver extract no. 343 and suitable amounts of a washed yeast suspension killed by heating for one-half hour at 80 to 85°C. Casein with autoclaved yeast extract could replace whole killed yeast (173). The larvae required at least two sets of growth factors, one present in liver extract, the other in yeast and yeast extract. The former set of factors has been studied in some detail (175). It was found that the liver extract contained a number of substances essential for the growth of *A. aegypti* larvae, one of them being riboflavin (176). In the presence of all the other growth factors supplied in partially purified form, crystalline riboflavin permitted normal larval growth at concentrations as low as 0.0004 mgm. per ml. and had a definite effect at a concentration of 0.00008 mgm. per ml. Thiamin, or certain of its degradation products, was also required. Pantothenic acid and vitamin B_6 are other essential factors (208). In addition to these organic growth factors, *A. aegypti* larvae require calcium (174), as do the larvae of *Theobaldia incidens* (53).

Frost et al. (53) studied the general nutritional requirements of *Theobaldia incidens*. When they attempted to rear larvae of this mosquito under sterile conditions in a heavy suspension of autoclaved bakers' yeast in distilled water, only about 25 per cent of the insects reached the adult stage. They did not try Trager's liver extract-killed yeast medium but instead did the rest of their work under non-sterile conditions. Hence no sound conclusions can be drawn from their results.

6. *Siphonaptera.* Dried blood alone or yeast alone did not support growth of the larvae of the rat flea *Nosopsyllus fasciatus*, but blood plus yeast enabled almost all of the larvae to reach the adult stage within 26 to 38 days (at 23°C.) (162). Both the serum and the red cells were essential, but hemoglobin or ferrous ammonium sulfate could be substituted for the red cells. The larvae grew on non-autoclaved red cells plus autoclaved serum plus autoclaved yeast, but did not grow if all the constituents were autoclaved.

7. *Hymenoptera.* It is well known that food is the factor which determines whether a female bee larva is to develop into a worker or into a queen. Larvae which receive the specially prepared food, "royal jelly," only during the first 3 days and then get honey and undigested pollen, develop into workers, whereas larvae which receive royal jelly

throughout the larval stage develop into queens. The problem as to what substances in royal jelly are responsible for this effect seems not to have attracted the attention it deserves. Aeppler (2) noted that royal jelly is high in protein and cystine and contains some vitamin B. The claim of Hill and Burdett (88) that royal jelly contains vitamin E appears to have been adequately refuted (134, 51).

Heyl (209) observed that the injection of pyridine extracts of royal jelly into immature rats increased the activity of the Graafian follicles, a result which Melampy and Stanley (210) could not repeat when they used acetone-dried royal jelly. Townsend and Lucas state in a preliminary report (211) that the ether soluble fraction of royal jelly when fed to *Drosophila* exerted a great influence on the rate of reaching sexual maturity and the number of eggs laid.

In connection with the problem of the effect of food on caste determination the remarkable results of Goetsch (68) with the ant *Pheidole pallidula* are worthy of note. Young, just fertilized queens of this ant lay small eggs from which only small, short-lived workers can develop, regardless of their food. Their fate is determined either by their genotypic constitution or possibly by the small amount of storage food in the eggs from which they hatch. The later, larger eggs can give rise to normal workers or to soldiers, and Goetsch found that the type of food determined which caste developed. Larvae fed pieces of insects, meat, or coagulated egg white developed into soldiers, while those fed sugar or honey developed into workers. Ant larvae given fluid protein such as blood, meat juice, or raw egg white developed into workers, as on sugar solution. Apparently a highly concentrated protein diet is necessary for the development of soldiers. Under natural conditions in a colony only a relatively few larvae would happen to find near them fragments of insects or meat. Consequently, only a few soldiers are developed, whereas the majority of the larvae get liquid food and become workers.

C. Requirements for maintenance and reproduction. Numerous species of insects can live in the adult stage and reproduce without taking any food at all. Others require only water, others carbohydrates, while others can live on a carbohydrate diet but must have other nutrients in order to reproduce. Still others appear to require a complex diet for maintenance as well as reproduction. In general, the greater the degree of development of the sex organs at the time the insect becomes adult, the simpler are its nutritional requirements during the adult stage.

Among the Lepidoptera, most species possess in the adult stage no

proteolytic enzymes whatever and frequently no enzyme other than invertase (163). In the latter group, water rather than sugar may be the essential substance. For example, adults of the oriental fruit moth lived as long and laid as many eggs when fed water as when fed 10 per cent sucrose solution (167). This problem has been investigated in greater detail by Norris (145) working with moths of the genus *Ephestia*. *E. kuehniella* could live and reproduce perfectly without any water, while *E. cautella* and *E. elutella* females, even if kept in an atmosphere of high relative humidity, required water in order to attain maximum egg laying and longevity. The males of the two latter species lived longer with water than without it. Females of *E. kuehniella* and *E. cautella* lived longer on a diet of sucrose solution than on distilled water, but did not lay more eggs. The feeding of albumin solution to *E. cautella* females had the same effect as distilled water. Norris concluded that the moth fat body has two main reserve foods, one serving to develop the ovaries and the other to maintain life. The latter, but not the former, can be partially replaced or supplemented by a sugar diet. Fecundity is not increased by the prolongation of life after exhaustion of the former substance.

The adults of the common Diptera have more complex requirements for maintenance and especially for reproduction than do the Lepidoptera. Glaser (58) found that houseflies died in 1 to 2 days if given no food. The feeding of a protein diet such as bouillon, blood serum, or egg white increased the longevity but slightly. A diet of sucrose alone increased the longevity considerably, but very few eggs were laid. A diet of sucrose plus bouillon increased the longevity still more and a great many eggs were laid. Evans (50) obtained similar results with the fleshfly *Lucilia sericata*. Sugar was necessary for growth of the imaginal fat body and maintenance of life in both sexes, while meat was essential for the development of the ovaries. Dorman, Hale and Hoskins (42) similarly found that various species of fleshflies did not develop their ovaries on a diet of sugar and water. After as long as 6 weeks on this diet, the addition of fish meat resulted in egg production within 10 days, the normal preoviposition period. Casein with butter and yeast, soluble casein, blood albumin, and Lemco beef extract could not replace fish meat, and when these substances were added to a sugar and water diet no eggs were laid.

Adults of the apple maggot lived a month on a diet of sugar and water but laid very few eggs (203). When fed protein and water they lived only a few days, but when fed sugar, protein and water they lived

a month and laid numerous eggs. Of various proteinaceous substances tried, proteose-peptone and yeast gave the highest oviposition rates.

Some species of blood-sucking insects resemble *Lucilia* in that the females must have a protein-rich food in order for the ovaries to develop. Most female mosquitoes appear to require blood, but Huff (99) showed conclusively that some *Culex pipiens* were able to lay eggs without a blood meal and in one case without any food at all. At the same time Roubaud (159) and Boissezon (15) independently found that certain *Culex pipiens* females could lay a first raft of eggs without taking any food during the adult stage. These females have been found to belong to the so-called autogenous race of *C. pipiens* (160, 180, 132).

Among all species of blood-sucking mosquitoes other than autogenous *C. pipiens*, the females require a blood meal for egg formation and, in the case of hibernating forms, for fat body formation (86). The rôle of blood in ovulation has recently been studied for two species. Roy (161) observed that for the development by *Aëdes aegypti* of a first batch of eggs at least 0.82 mgm. of blood had to be ingested at one meal. Within limits, the number of eggs laid was approximately proportional to the size of the blood meal. According to Woke (186) washed red cells, serum, heparinized plasma, and hemoglobin solution from laked red cells, when fed separately, all supported normal egg production by *Aëdes aegypti*. If females of *Anopheles elutus* were given a blood meal 24 hours after emergence, only a few formed ripe eggs, while many reached Christophers' stage II of ovarian development (138). More produced ripe eggs after a second blood meal. In females fed raisins only, no eggs matured but the ovaries reached stage II. Such females, if given one blood meal after 7 days on the raisin diet, formed mature eggs in practically all instances. The feeding of washed red cells in saline induced maturation of the eggs of *A. elutus* (193). If the females were fed hemoglobin solution the ovaries reached stage II but no ripe eggs formed; if they were fed serum the ovaries did not even reach stage II. However, if insects previously fed raisins or sugar, and hence with their ovaries already in stage II, were fed serum the ovaries matured, whereas if they were given hemoglobin solution the ovaries did not mature. Hemoglobin solution, like sugar, raisins, etc., can bring the ovaries only to stage II. In order for the eggs to mature, another specific factor, present in serum and in the stroma of red blood cells, is required. The factor in serum resisted heating at 60°C. for 1 hour and was only partially inactivated by 30 minutes at 100°C. Heating serum at 100°C. for 15 minutes produced a coagulum which was found to

contain the activity. Ether extraction of serum did not remove the active substance. It is interesting to note that Glaser (58) had obtained similar results with the biting fly *Stomoxys calcitrans*. This insect could be fed on drops of defibrinated blood previously warmed to 35 to 37°C. On this diet the flies lived 20 days or more and laid many eggs. On serum alone or blood cells alone the longevity was lower and no eggs were laid. Recombination of the two blood fractions gave normal longevity and oviposition.

It is a remarkable fact that intracellular microörganisms or symbionts (18) occur in all insects and related arthropods which feed exclusively on blood throughout the life cycle. The Triatomidae, which were formerly considered to be an exception to this rule, have been shown (183) to contain organisms of a diphtheroid type which inhabit certain cells of the alimentary tract. Few of the insects which feed on blood during only a part of their life cycle contain such symbionts. Sterile blood is an insufficient diet for mosquito larvae and fleshfly larvae, whereas blood infected with bacteria is adequate. These facts led Wigglesworth (181) and Aschner (4) independently to suggest the hypothesis that the symbionts of the strictly blood-feeding forms furnish essential accessory growth substances. The only direct evidence for this is that of Aschner and Ries (6), and Aschner (5), who showed clearly that removal of the symbionts of the body louse, *Pediculus corporis*, resulted in cessation of growth and death in young lice and grave interference with reproduction in adult females. The experiments were so designed that it was evident that the procedures used to remove the symbionts were not in themselves deleterious. Some evidence was also obtained indicating that the deficiency produced by removal of the symbionts could be partly compensated by the intrarectal feeding of yeast extract.

Haydak (82, 83, 85) observed effects of food on the activities of adult worker honey bees (*Apis mellifica*). The nitrogen content of the young worker bees had to increase to a certain level before brood rearing began. When bees which had started brood rearing on a normal diet were confined to a diet of sugar and water, normal nursing activity continued for only one week. All the larvae fed after that period were unable to mature and were removed by the workers, the nitrogen content of which was considerably lower than at the beginning of the experiment. Bees in experimental colonies which were fed honey with pollen, meat scrap, or commercial casein developed their thoraces with respect to dry weight and nitrogen content at about the

same rate and to the same extent. On cottonseed meal, the dry weight increased normally but the nitrogen increase was slower. On dried blood, digested tankage, whole wheat flour, whole oats flour, corn flour, or pea flour the increase in dry weight and nitrogen was much below normal. On meat scrap and cottonseed meal the mortality was almost as low as on pollen, but it was much higher on the other foods. Brood rearing started on the three best foods of meat scrap, cottonseed meal, and casein, but was carried through only on the first of these diets. On all the other foods the larvae were removed from their cells at an early age. Removal of the queen from such colonies did not stimulate brood rearing. The degree of brood rearing activity on the different foods corresponded in general to their richness in protein, potassium, phosphorus, and sulfur.

III. Other invertebrates. So little is known concerning the nutritional requirements of invertebrates other than protozoa and insects that they can be conveniently considered in one section in a taxonomic order. Work concerned exclusively with digestive enzymes will not be reviewed.

A. Coelenterata. Hammett and his associates (77–81) have studied the effects on *Obelia geniculata* of feeding various pure substances known to be among the constituents of living cells. Sulfhydryl stimulated the proliferation of new hydranths, but did not affect differentiation. Glycine specifically accelerated regeneration; alanine had no effect; phenylalanine slightly favored differentiation; glutamic acid hastened differentiation and organization; tryptophane retarded catabolism; histidine similarly acted as an indirect stimulus to general growth by its sustaining effect on metabolic expenditure; d-arginine favored recurrent growth and retarded differentiation, regeneration, and metaplasia. Uracil, hypoxanthine and xanthine had the same effect as d-arginine; d-ribose had no effect; allantoin favored growth in general; adenine favored organization but retarded new growth formation.

B. Plathelminthes. The nutrition of *Planaria maculata* has been the subject of a number of interesting investigations. Wulzen (188) found that growth was less on heated mammalian tissue fragments than on unheated ones. The heat labile factor could be extracted with ether (189). The eosinophilic portion of beef pituitary had much more growth promoting power than the basophilic portion (190). If egg white was incorporated into an already adequate diet of liver pulp and starch it exerted a toxic effect when present in certain proportions (191). Wulzen and Bahrs (192) fed guinea pigs for one month on

adequate diets in which vitamin C was supplied to three different groups as: 1, orange juice; 2, tomato juice; 3, fresh grass or fresh kale. The fresh tissues of the guinea pigs were fed to planaria. On spleen from all groups of guinea pigs the worms remained healthy and grew rapidly. On lung they remained healthy and grew fairly well. On liver, good growth occurred only among the worms given liver from guinea pigs which had been fed kale. The next best growth was obtained on the livers of grass-fed guinea pigs. On the livers of guinea pigs fed orange juice, and more especially tomato juice, the planarians showed by the third week a deficiency disease consisting of decreased growth, roughening of edges, development of irregular lumps, darkening of color, and a twisting out of shape. Similar results were obtained with heart and kidney. The planarian disease did not develop on these organs taken from guinea pigs which were fed both kale and tomato juice, showing that there was no toxic effect of the juice. The guinea pigs on the different diets all appeared equally healthy. It may be noted, however, that grass does contain substances, other than the recognized vitamins, which are essential for the growth of guinea pigs (108). Bahrs and Wulzen (8) have also found that spleen, lung, liver, heart, or kidney, from guinea pigs kept on diets deficient in any of the known vitamins, had a lower growth promoting power for planaria than did tissues from normal animals. Greenberg and Schmidt (70) found in liver a heat labile, ether soluble factor which, if added to a lean beef diet, markedly increased the growth of *Planaria maculata*. The substance could not be replaced by liver phosphatide, cephalin, lecithin, sphingospongin, sterols, known vitamins, unsaturated fats, fatty acids, auxin, or aqueous liver extract. The factor was present in whole yeast. It resembled in many respects McCay's factor H for trout (126).

C. Nemathelminthes. The free-living nematodes can be readily cultured on various media if living microörganisms, especially bacteria, are present. Metcalf (140) sterilized the eggs of *Rhabditis brevispina* by washing in sterile water and plating out on agar. In a sterile asparagus juice agar, larvae which hatched from sterile eggs were able to grow only past the first molt. But sterile larvae, hatched out in a sterile filtrate from a *Fusarium* culture, developed rapidly and normally.

The vinegar eel, *Anguillula oxophila*, can develop under natural conditions in various fermenting media, and is known to feed on bacteria. Zimmerman (198) succeeded in freeing worms of this species from microörganisms by repeated treatments with peroxide and sterile water. Such sterile worms, if placed in sterile vinegar, lived 11 to 16

days but did not grow. They also did not grow on autoclaved flour paste. The worms did grow under sterile conditions in a medium prepared by allowing flour paste to putrefy, filtering it, and autoclaving the turbid filtrate. Sterilized worms placed in sterile 2 per cent peptone solution with 0.5 per cent sodium chloride, or in beef broth, lived for some time but never reached sexual maturity. The addition of lecithin gave no improvement. In a medium consisting of yeast autolysate and peptone the worms lived for months but did not reproduce. If lecithin was added to the medium, they soon began to multiply. A medium exactly like that used by Guyénot for *Drosophila* larvae (see section II, *Diptera*), consisting of peptone, salt, lecithin, and an alcoholic extract of yeast autolysate, gave rapid growth and multiplication of the worms.

Glaser (59) succeeded in culturing *Neoaplectana glaseri*, a nematode which is taxonomically closely related to the free-living forms, but is parasitic in Japanese beetle grubs. The nematode was grown on plates of dextrose veal infusion agar covered with a growth of living yeast. Chance-mixed bacteria were present. Under such conditions of culture, three to four generations developed in a period of two weeks before transfer to fresh medium was necessary. After about six such transfers (18 to 24 generations) the female worms apparently became sterile and the cultures consequently died out. They could be saved by infecting live beetle grubs with them. After several grub passages, the worms could again be cultured *in vitro* for 18 to 24 generations, when they would again die out. It was then found (212) that if the cultures, just before they began to die out, were supplied with a powder made from dried beetle grubs, or with powdered whole bovine ovarian substance, they revived. In the presence of the ovarian substance the worms have been kept in culture continuously for several years and apparently can be kept indefinitely. Various proteinaceous substances which were tried could not be substituted for ovarian powder or beetle powder. The active substance in the ovarian powder was destroyed by sterilization in the autoclave but not by Arnold steam sterilization. In the absence of the ovarian powder the cultures waned, apparently because of a gradually accumulated deficiency of a nutritional factor essential for reproduction.

Study of the nutritional requirements of this parasite of Japanese beetles will be facilitated by a method, recently developed, for their culture free from microörganisms (213).

The free-living stages of the parasitic nematodes of vertebrates, like

the free-living nematodes, also feed on bacteria. McCoy (129) found that larvae of the dog hookworm, *Ancylostoma caninum*, developed to the infective stage in pure cultures of various species of bacteria and in saline suspensions of live bacteria, but not in the presence of killed bacteria only. Glaser and Stoll (65) were able to rear to the infective stage, under sterile conditions, the larvae of *Hemonchus contortus*, the sheep stomach worm. The medium consisted of a fragment of fresh sterile rabbit kidney, heat-killed ground yeast, and liver extract (Eli Lilly no. 343) all incorporated in a soft semi-solid agar gel.

In a somewhat similar but more complex medium, Glaser and Stoll (66) have also obtained partial development of the parasitic stages of *Hemonchus contortus*. Before this work was accomplished, no helminth parasite of a vertebrate host had ever been induced to grow anywhere outside of a living animal.

D. Mollusca. It is now generally admitted that diatoms and other minute marine forms, including small animals (144), are the chief food of oysters and related Lamellibranchs (143, 194, 195, 25). The nature of the substances which they must obtain from this food is quite unknown. A beginning toward a more detailed study of the nutritional requirements of oysters was made by Martin (133), who fed oysters on pure cultures of several species of plankton organisms and found that the oysters grew better on some than on others.

Howes and Whellock (98), in the only study of its kind conducted with a mollusc, found that the snail *Helix pomatia* did not grow on a diet containing casein, glucose, lactose, starch, dried yeast, cystine, calcium carbonate, a complete salt mixture, filter paper, and lemon juice unless this was supplemented with both cholesterol and leaf extract or cod liver oil. Since the cholesterol was not quite pure, it may have been supplying some other sterol which was actually the essential substance. There was little growth if the dried yeast was omitted from the otherwise complete diet.

E. Arthropoda. Crustacea. The Cladocera, which have proved useful in various types of experimental work, are ordinarily reared in media, such as manure infusion, which are rich in microörganisms. Stuart, McPherson and Cooper (165) freed adult female *Moina macrocopa* from contaminating microörganisms and studied their nutritional requirements. The Cladocera grew well in pure cultures of live bacteria. In sterile water the *Moina* died without producing young. In autoclaved pond water, in Berkefeld-filtered or autoclaved normal medium, and in broth, 30 to 40 per cent of the *Moina* produced young. If killed

Bacillus coli were added to any of these media 40 to 50 per cent of the *Moina* produced a first clutch of young. In no case were continuous growth and reproduction obtained under sterile conditions. Similar results were obtained by Gellis and Clarke (57) with *Daphnia magna*.

Viehoever and Cohen (178) found that the growth, ovarian function, and reproduction of young parthenogenetic females of *Daphnia magna* were greatly inhibited in a medium made from sheep manure which had been extracted with petroleum ether. If small amounts of Triticol (cold-pressed wheat germ oil) were added to the same medium, growth and reproduction were normal. The authors interpret these results as indicating a need of *Daphnia* for vitamin E. Since microörganisms were present, the effect may have been an indirect one via some effect on the microörganisms. Moreover, Triticol contains a number of substances, other than vitamin E, which might have been the effective factors.

IV. General discussion. Knight (104) classified bacteria in groups having decreasing synthetic abilities, a type of classification already suggested by A. Lwoff (113) for microörganisms in general. It is of interest to attempt to place the invertebrates in Knight's scheme. His first main group consists of the autotrophic bacteria which assimilate nitrogen from inorganic sources and carbon from carbon dioxide. They obtain energy for the reduction of carbon dioxide either from light or from the oxidation of simple inorganic compounds. With the exception of the still very doubtful case of *Chilomonas paramecium*, no invertebrate falls in this group. Bacteria of the second group obtain their energy and carbon for assimilation from carbon compounds already more reduced than carbon dioxide, which is not assimilated. They derive their nitrogen from simple inorganic compounds. In this group could be included only a few of the colorless plant-like flagellates. Bacteria of the third group differ from those of the second only in requiring their nitrogen already combined as an amino acid, while those of the fourth group require, like vertebrates, a whole array of amino acids and also accessory growth-promoting substances. It is apparent that, at least on the basis of our present knowledge, all of the invertebrates except the colorless plant-like flagellates fall in this fourth group. Even the simplest free-living ciliates require numerous amino acids and at least one growth factor (thiamin). The metazoan invertebrates which have been studied have nutritional requirements at least as complex as those of vertebrates or of the most highly parasitic bacteria and protozoa.

Hence both the similarities and differences between the nutritional requirements of vertebrates and invertebrates have a great significance. Organisms of neither group can synthesize thiamin or riboflavin. On the other hand, vertebrates can synthesize cholesterol but not vitamin D, whereas certain invertebrates cannot synthesize cholesterol. Whether these organisms can synthesize vitamin D is not known. Perhaps in them cholesterol is somehow concerned with calcium and phosphorus metabolism, as the related sterols of the vitamin D group are in vertebrates. A knowledge of the function of cholesterol in the growth of organisms which require it as a nutrient, a knowledge of the rôle of organic acids in the growth of certain flagellates, or of the nature of the nutrient substances which affect caste determination in the social Hymenoptera, in short, a more penetrating knowledge concerning the nutritional requirements of invertebrates cannot help but be important to a better understanding of the general physiology of organisms.

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THE SECRETIONS OF THE INTESTINE

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For the purpose of this review the intestine is divided into three parts which have clear cut structural differences and which have distinctive functions to perform: *A*, the part of the duodenum just distal to the pylorus, containing the duodenal or Brunner's glands; *B*, the rest of the duodenum, the jejunum and the ileum; *C*, the cecum and colon.

The review is concerned only with mammals, and with the external secretions of the intestine.

A. THE BRUNNER'S GLAND AREA. *Anatomy.* The first part of the duodenum has embedded in its mucosa and submucosa the duodenal glands. These structures, which in many species appear to be continuous with the pyloric glands (for extensive discussion of this and other histological points see Villemin (1922), Plenk (1932), Patzelt (1936)), occupy both the mucosa and submucosa near the pylorus, but as they pass distally soon come to lie wholly beneath the muscularis mucosae. They are composed of ramifying tubules which empty by many ducts into the bases or sides of the crypts of Lieberkühn in the overlying mucosa. The cells which make up the alveoli of the glands are histologically of the mucous type, though in the rabbit and hare "serous" cells are also present (Tschassownikow, 1928a, b; Haase, 1937). Villemin (1922) states that they are also present in the horse.

Characters of the juice. Colin (1854), by his acute experiments on the horse, seems to have been the first to collect and accurately describe duodenal juice. Although extracts of the upper part of the duodenum were investigated by a certain number of subsequent workers, its secretion was not examined again till 1902, when Ponomarew (1902), working in Pavlov's laboratory, used fistula methods. He described the secretion from the first part of the duodenum of two dogs as a colorless syrup-like fluid, composed of a clear, fluid part and light gray mucus.

Recently the secretion of the upper part of the duodenum was extensively reinvestigated (Florey and Harding, 1933a, 1934, 1935a,

b; Wright *et al.*, 1940). The physical characters of the juice were usually those described by Ponomarew, though some of his conclusions on the control of the secretion were not substantiated. Utilizing both "acute" preparations and fistulae of various types, the secretions were collected from the cat, dog, goat, sheep, rabbit and pig. No differences were found between the juices from denervated and innervated fistulae (pig, cat, rabbit). From good clean fistulae the juices were clear, either colorless or faintly yellow. Deposit was often inappreciable even after centrifuging but when present consisted of grayish mucin (probably from the goblet cells of the villi) and cell débris, for the most part of unrecognizable origin though no doubt much of it epithelial. Bacteria were also found.

The specific gravity varied between 1007 and 1009. The juices from all the five species were mucoid, but the degree of stickiness varied from species to species. Juice from fistulae of the rabbit, goat and sheep was thicker and more like egg-white than that from the pig, cat and dog, which pulled out into long tenacious threads. Acetic acid precipitated the mucin in the first group but only produced a faint haze in the second. It was shown that both types of mucin produced large amounts of reducing substances when hydrolyzed, one of the accepted chemical characteristics of true mucins. In parenthesis, it may here be remarked that very little is known of the chemical composition and ultimate fate of secreted mucin in the gastro-intestinal tract; the subject might well repay careful investigation.

Chemical composition. Ponomarew (1902) noted that dog's duodenal juice was alkaline and estimated that the alkalinity was equal to that of 0.09 to 0.15 per cent Na_2CO_3. Florey and Harding (1933a, 1934) investigated the juice of several species and found that that of the goat had very little neutralizing power (1 cc. equivalent to 0.04 cc. N/10 soda), but that 1 cc. of rabbit fistula juice was equivalent to as much as 0.75 cc. N/10 soda. Rabbit juice obtained in acute experiments gave even higher figures (e.g., 1 cc. equivalent to 1.32 cc. N/10 soda). The values for cat, dog and pig juice lay between those for the goat and rabbit, 1 cc. being equivalent to from 0.25 to 0.5 cc. N/10 soda. All these juices had a very alkaline pH at the end of the collection period, no doubt partly due to the loss of CO_2 to the air during collection. Rabbit juice sometimes became as alkaline as pH 9.3 but when it was collected under oil values of pH 8.0 to 8.2 were obtained. The pH of goat juice was 8.2 to 8.4, pig juice 8.4 to 8.9, cat juice 8.7 to 8.9, dog juice 8.4 and sheep juice 8.3 to 8.4, when collected in contact with air. That the

buffering power of the juice was mainly dependent on bicarbonate and not on mucin was shown by Havard (1934).

The total solids of fistula juices varied from 1.5 per cent for the goat to 1.2 per cent for the pig, slightly more than half the solids being inorganic. No detailed examination of the mineral content was made but there appeared to be considerable amounts of sodium chloride present. Mucin, no doubt, makes up the greater part of the organic solids.

Enzymes. The duodenal enzymes have been investigated by making extracts of the mucosa and by examining the secreted juice. For determining what enzymes are actually secreted the former method is useless, although enzymes destined to be secreted into the lumen may, among others, be found in the mucosa. Ponomarew (1902) reported that the juice contained invertase, lipase and a feeble or inconstant amylase, as well as pepsin-like and rennin-like enzymes; the presence of the last two was confirmed by Pavlov and Parastschuk (1904) and of the pepsin-like enzyme by Abderhalden and Rona (1906). Florey and Harding (1934) reported the presence of a pepsin-like enzyme in goat and dog juice and a trace of lipase in the dog. Ponomarew found enterokinase.

A comparative investigation was recently made (Wright *et al.*, 1940) on the enzyme content of spontaneously secreted juice from a number of species and also on that of juice collected after vagal stimulation, sympathetic section and eserine administration in the cat. Care was taken to avoid bacterial contamination and to keep the cellular content to a minimum. In addition good chemical methods were used. The tests for proteases were consistently negative; no trace of a pepsin-like, trypsin-like or cathepsin-like enzyme was ever found. Tests for dipeptidase and polypeptidase were nearly always negative; significant splitting of these substrates ran parallel with exceptional cellularity of the juice. There was no evidence that cell-free juice contained significant amounts of invertase. Traces of lipase were occasionally found. The importance of the cellular content was indicated by the fact that addition of intestinal villi to juice which was negative for dipeptidase and invertase gave it the property of splitting dipeptides and cane-sugar.

Whatever their method of production, the juices always contained amylase. With a very few unexplained exceptions they were also capable of fully activating pancreatic juice to digest protein at an alkaline pH (enterokinase). In this connection it is noteworthy that Kunitz

(1938–39) used the secretion present in the duodenums of pigs after slaughter as a source of material for purifying enterokinase.

A bacteriolytic enzyme, lysozyme, discovered by Fleming (1922) was also found in appreciable quantities in pig juice, but only in traces in the other species tested.

CONTROL OF SECRETION. It should be emphasized that in all species the typical mucoid juice could be collected without subjecting the fistula to mechanical stimulation, e.g., by a catheter. This sharply differentiates fistulae of the upper duodenum from those of other parts of the intestine, which, except for small amounts of "periodic" juice (Boldyreff, 1904, 1928), will only produce fluid after some form of local stimulation.

Effect of local stimulation. Ponomarew (1902) showed that the introduction into the fistula of undiluted gastric juice, 0.25 per cent HCl, the products of the digestion of fibrin by gastric juice, one drop of mustard oil in 200 cc. water, and even normal saline increased the flow of juice. Fatty substances gave little or no increase. His work was extended by Florey and Harding (1933 a, 1934) who investigated the effects of perfusing dilute HCl (usually N/10 or less) through the first part of the duodenum and found that the fluid increased in volume during its passage and became mucoid. A considerable degree of neutralization also occurred. Histological evidence was obtained from the cat, rabbit and guinea pig that the passage of HCl exhausted the mucin-containing cells of the duodenal glands. The neutralization and the production of mucus were not inhibited by atropine. The perfusion of N/20 H_2SO_4 also caused secretion, but when dilute mustard oil (one drop in 100–150 cc. water) was perfused fluid was only produced concurrently with the histological picture of inflammation.

The influence of food. Ponomarew (1902) reported that feeding did not influence the flow of juice from duodenal fistulae in the dog. Cats, dogs and pigs (Florey and Harding, 1934, 1935a, b; Wright *et al.*, 1940) have lately been investigated. When starved for 24 hours the fistulae ceased to secrete or only produced a few drops an hour; within a few minutes of taking food juice began to flow. A similar phenomenon was observed in a goat. Later the same was shown in cats and pigs, in duodenums which had been transplanted subcutaneously and deprived of all nervous and vascular connections with the abdominal contents. A similar denervated fistula in the rabbit secreted juice continuously, probably because the stomach of the rabbit is never empty. Clearly, in the cat and pig at least, some form of hormonal

stimulus was at work. Highly purified secretin injected intravenously in the cat caused typical secretion from a quiescent fistula. Cruder secretin preparations were active on innervated fistulae in the dog.

Fogelson and Bachrach (1939), working with dogs, confirmed the finding that upper duodenal fistulae secreted a mucoid juice, the volume of which was increased by a mixed meal. They too found that secretin given intravenously produced a flow of mucoid duodenal juice; they showed that the flow was accompanied by muscular contractions and their conclusion was that no proof had yet been offered of such secretion being independent of muscular action.

Ponomarew (1902) reported that 0.5 per cent HCl inserted into the stomach increased the secretion from a dog fistula slightly, while olive oil and 5 per cent and 10 per cent solutions of sodium oleinate increased the secretion markedly. When these experiments were repeated on cats (Florey and Harding, 1935b), olive oil and cane sugar did not cause secretion but meat and milk invariably caused a flow of juice within 3 to 4 minutes of ingestion. The insertion of N/10 or N/5 HCl into the stomach also produced juice but the amount was never as much as after meat and milk.

The influence of nerves. It was recently shown (Wright *et al.*, 1940) that prolonged stimulation of the infracardiac vagi of decerebrate or decapitate cats regularly produced a considerable flow of mucoid juice from the first part of the duodenum. This juice was indistinguishable in its physical characters from that collected from a permanent fistula, whether innervated or denervated, and its production was accompanied by histologically demonstrable exhaustion of the mucus-containing cells of Brunner's glands. This outspoken effect of vagal stimulation was abolished by certain of the barbiturate anesthetics and all anesthetics tested had some inhibitory effect.

In addition to the vagal effect, it was found that cutting the greater splanchnic nerves in the thorax was followed by secretion of typical mucoid juice from the first part of the duodenum. Large doses of eserine subcutaneously gave a similar secretion; if acetyl choline was given to an eserinized cat pieces of mucosa were often shed into the juice.

It appears from these results that the part of the duodenum containing Brunner's glands is controlled by a hormone, possibly secretin, and has in addition a sensitive acetyl-choline mechanism which can be set in motion by vagal stimulation, by the removal of a sympathetic nerve inhibition or by paralyzing the esterase with eserine. The fact that

pilocarpine produces juice from the duodenum is supporting evidence for the participation of the vagus in the control of the duodenal secretion.

It has been stated that the administration of histamine causes a flow of intestinal juice. In the first part of the duodenum this drug appeared to be without effect.

The amount of juice secreted. Ponomarew (1902) stated that his two dogs produced 0.23 to 1.3 cc. an hour and 0.06 to 1.08 cc. an hour respectively. Florey and Harding (1934) obtained a steady 1.5 cc. an hour from a dog after food while Wright *et al.* (1940) obtained 1.5 cc. and 2.0 cc. an hour respectively from two fully innervated fistulae in dogs. Fogelson and Bachrach (1939) got as much as 8.8 cc. in an hour from a fed dog. From cat fistulae 0.25 to 0.5 cc. an hour was obtained. Relatively much larger quantities of juice are secreted by pigs, goats and rabbits, which have a greater development of Brunner's glands. From one pig as much as 17.0 cc. an hour was obtained and this from a fistula which did not by any means contain all the Brunner's glands. One large goat secreted 150 cc. in 7 hours, though most goats secreted considerably less than this. In rabbits, too, there was a relatively large production of duodenal juice though no fistula included more than a small part of the Brunner's glands. It might be thought that the continuous loss of some or all of the duodenal secretion from these fistulae would influence the general health of the animal, but no interference with growth or health was observed.

The quantity of juice produced from the duodenum in experiments on decerebrate or decapitate cats was considerably greater than that obtained from cat fistulae. A longer piece of duodenum was used in most of the acute experiments but there is good evidence that the juice came mainly from the upper 2 cm. (i.e., the Brunner's gland area). The quantities of clear sticky duodenal juice obtained from these acute preparations varied from 2 to 8 cc. per hour. Possibly a fistula never receives maximal nervous stimulation and therefore never secretes to full capacity. There is good evidence that a satisfactory fistula has a histologically normal mucosa, at least for several weeks, and an adequate blood supply. Volta (1920) reported on two Thiry fistulae of the intestine which he considered were functionally and anatomically normal after five years.

The duodenum and hemopoiesis. Meulengracht (1935), basing his work on Castle's (Castle and Minot, 1936) and Sturgis and Isaac's (1930) elucidation of the gastric factors involved in pernicious anemia, showed that extracts of that part of the duodenum containing Brunner's

glands were active in supplying "intrinsic factor" for the treatment of pernicious anemia. He suggested that the cardiac part of the stomach, the pyloric antrum and the first part of the duodenum, which have certain histological similarities, formed a "gland organ" which secreted the factor. Thompson (1937) also found that duodenal extracts were effective, though he did not say whether he thought Brunner's glands were responsible for the activity.

No one has yet shown whether juice secreted by the pig's duodenum (probably mainly from Brunner's glands) contains the "intrinsic factor."

Goodfriend et al. (1938) obtained a polypeptide preparation from the duodenal juice of pigs and goats which gave a reticulocyte response in the guinea pig (Jacobson's test). Their preparation was active in extremely small doses (e.g., 0.04 μg) and gave a sufficiently appreciable and constant response for assay purposes. Their material was, however, inactive in clinical cases of pernicious anemia and its function in hemopoiesis, if any, has not been elucidated.

Production of secretin. Volborth (1925) showed secretin to be present in the débris of intestinal juice, but it is unlikely to be secreted into the lumen of the intestine and then reabsorbed. The fact that secretin is only active when administered intravenously is against the view that it is secreted with the duodenal juice.

Functions of duodenal juice. As the result of the investigations of Ponomarew (1902) and Dobromyslow (1903) it was suggested that one of the functions of duodenal juice was to assist in the digestion of fats by providing a proteolytic enzyme, active in acid juice, which would help to remove the connective tissue covering from fat-containing cells. It is difficult to see how such a ferment could act in the first part of the duodenum, especially as bile was said to inhibit the proteolysis in certain circumstances, and later investigations have failed to substantiate the presence of a proteolytic enzyme. As far as is at present known the only enzymes constantly present are amylase and enterokinase. The amylase is probably weak compared with pancreatic amylase (though no exact figures are available) and therefore not of much significance. The enterokinase, on the other hand, appears to be powerful, and it is no doubt of the greatest importance in activating the pancreatic juice as it emerges into the intestine. Whether duodenal juice contains more enterokinase than juice from the rest of the small intestine, as Pavlov believed, has not yet been determined.

It has long been thought that mucous secretions, by virtue of the physico-chemical properties of mucins, protect the underlying mucosa.

The alkalinity and high mucin content of duodenal juice suggest that the secretion of Brunner's glands may protect the delicate villi of the first part of the intestine from the damaging action of the ejected acid stomach contents. The possibility is supported by experiments on pigs (Florey *et al.*, 1939) in which it was shown that duodenum containing Brunner's glands was more resistant to gastric juice than other parts of the small intestine.

Villemin (1922), after studying the comparative anatomy of Brunner's glands, pointed out that with few exceptions they ceased at the entrance of the pancreatic duct but were independent of the entry of the bile duct. He inclined to the view that they took part in the neutralization of gastric juice.

This mucoid duodenal juice may also be useful in suspending food particles during intestinal digestion. Such a function appears particularly likely in the rabbit, where the chyme issuing from the pylorus is considerably diluted by the duodenal secretion (Florey and Harding, 1933b).

The possible function of the juice in hemopoiesis has already been mentioned.

Regarding the special contribution of Brunner's glands to the duodenal juice, it may be said that while the Brunner's gland area gives a good secretion in response to vagal stimulation, the adjacent few centimeters of the duodenum give a scanty secretion, and the rest of the small intestine none (in the cat; see section B). Similarly, in response to feeding, only fistulae which include the Brunner's gland area secrete spontaneously. Brunner's glands may therefore reasonably be held responsible for a large part of such secretions. There are good grounds for believing that the mucin is their product. It is impossible, however, to say precisely what are the contributions of the Brunner's glands on the one hand, and of the villi and crypts of Lieberkühn on the other, to the normal duodenal juice.

B. Small intestine. *Anatomy.* The mucosa lining the small intestine consists of tubular glands—the crypts of Lieberkühn—and projecting villi. Both are invested by a single layer of epithelial cells with other cells interspersed between. The investing layer consists of simple cuboidal or cylindrical cells and goblet cells, the latter reaching their fullest development on the villi; judging by the position of the many mitoses these cells all originate in the lower two-thirds of the crypts. Argentaffine cells scattered between the epithelial cells are found mainly in the upper part of the intestine; Paneth cells—cells with

large acidophil granules—are present at the bases of the crypts in some species, while the many small cells which lie between the epithelial cells are almost certainly lymphocytes. (The histological picture is discussed at length by V. Patzelt, 1936, p. 74, and the reader is referred to his article.) Our knowledge of the histology of the mucosa, which is considerable, contrasts with our lack of knowledge of the function of its several component cells. It has not, for instance, been settled whether the crypts secrete and the villi absorb or whether they have a common function, as the common origin of their epithelial covering might be taken to indicate, in which case both crypts and villi would secrete and absorb. Still less is anything certain known about the contribution, if any, of the Paneth and argentaffine cells to the secretion. The most that can be said about the functions of the lymphocytes is that some workers see in them a defence against harmful substances in the intestine (Hellman, 1934), though it has recently been shown that all the Peyer's patches can be removed from a rabbit or a rat without detriment to the health of the animal and without the formation of compensatory nodules (Sanders and Florey, 1940).

Experimental methods. Nearly all experiments have been done on the dog, and it is to be regretted that more is not known about the intestinal functions of other species. Two kinds of experiments have mainly been used—acute preparations, in which a loop of intestine is examined for a short time, and "chronic" or permanent fistulae, where the lumen of a loop of intestine is isolated from that of the main gut and made to empty externally.

Frerichs (1846) claimed that he was the first to use the acute preparation, and some of the latest workers have used it again. Chronic preparations have changed little, except for the general improvement in operative methods, since Thiry (1864) described and Vella (1888a) modified the fistula operation; preparations almost identical with theirs are generally used at the present time, though Johnston has described a modification which seems to have some advantages. Several kinds of fistulae, some not much used now, were devised in Pavlov's laboratory. Details of preparing the fistulae may be found in Markowitz (1937), Johnston (1932–33) and Babkin (1928, p. 760–762), who also gives further references.

Pavlov claimed to have made a fistula which was fully innervated. The interference needed to close the lumen, however, inevitably interrupts the submucous plexus. In all fistulae of the Thiry and Thiry-Vella types the muscle is cut, thus interrupting the myenteric plexus

as well; such fistulae are possibly deprived of at least part of their vagal supply but, at any rate while the pedicle remains healthy, retain their sympathetic supply.

SECRETION BY A FISTULA. *Spontaneous secretion.* It is an almost constant finding that fistulae of the jejunum or ileum do not secrete spontaneously in response to feeding. Boldyreff (1904), however, described a "periodic" spontaneous secretion, occurring at intervals of from 1 to 2 hours in starving dogs and inhibited by food. A fistula 20 to 30 cm. long would secrete up to 2 cc. of juice in 20 to 30 minutes and therefore, Boldyreff calculated, the whole intestine secreted about 30 cc. of juice at each period. Leper (1904), Brynk (1911) and Komarow (1924a) corroborated Boldyreff and the last noticed that atropine inhibited the secretion. Babkin and Ishikawa (1912) believed that the periodic secretion was related to increased gut movement. Boldyreff (1928) recently reaffirmed his opinion that periodic juice was a true intestinal secretion.

Response to local stimulation. In his original paper Thiry (1864) described how mechanical stimulation of an intestinal fistula by a sponge or catheter produced a flow of fluid, and at the present time an ordinary catheter or some modification such as Johnston's, is commonly used for obtaining fistula juice. Physiologists seem to accept such mechanically produced juice as true *succus entericus* though, as Boldyreff (1928) pointed out, it often contains blood. He considered it a pathological transudate, but he was not very consistent, as there is little or no material difference in his figures for the enzyme content of spontaneous periodic secretion and of juice produced by "gentle" stimulation. Pavlov (1910) regarded the *succus entericus* as a fluid whose composition was determined by the stimulus to secretion. Thus a mechanical stimulus excited a secretion of water to wash away the foreign body, and specific stimuli promoted the secretion of enzymes, notably pancreatic juice the production of enterokinase. This last was re-affirmed by Savich (1921). Similarly, Lombroso (1908) thought that fat and bile-salts, and Jansen (1910) that bile-salts alone, were the stimulus for lipase, but Orbéli (1917) could not confirm Jansen, and London and Dobrowolskaja (1910) denied that this kind of adaptation of enzyme to stimulus occurred.

Many chemical irritants have been found effective, e.g., acetic acid, 0.1 per cent HCl, 20 per cent $MgSO_4$, 0.25 per cent butyric acid, gastric secretion, mustard oil emulsion, triolein, gliadin albumose, lactose, erythro-dextrin, sodium oleate, ether, chloral hydrate, calomel (Babkin,

1928, p. 772). All of these substances except gastric juice have been used in much higher concentrations than are likely to occur normally.

Electrical stimulation apparently also causes some secretion. Dobroslawin (1870) described stimulating for ¼ hour periods with an electrode inside the fistula and collecting from 2 to 3 times as much juice during stimulation as during rest. A few other similar reports have been given (Thiry, 1864; Masloff, 1882; Bastianelli, 1892).

Characters of fistula juice. There is general agreement that the juice from a fistula consists of two parts—a watery supernatant fluid and a clumpy or floccular part. The fluid is slightly yellow, is clear or opalescent, has an aromatic smell and an alkaline reaction. The floccules are mainly composed of mucus with which are mixed degenerating epithelial cells, microörganisms and cholesterol crystals.

Amount of juice collected. The amounts which can be collected from a fistula by mechanical stimulation vary widely, even in one animal. De Beer *et al.* (1935) obtained from 64 to 175 cc. from a jejunal fistula in one dog, and from 10 to 60 cc. in another, in two hour collection periods; an ileal fistula yielded about 65 cc. in the first two hours of collection. Mosenthal (1911) holds the record with 115 cc. in one hour from an ileal fistula between 25 and 50 cm. long. Many workers have never obtained more than a few cubic centimeters (e.g., Andrejew and Georgiewsky, 1932; Nasset *et al.* 1935).

Effect of denervation. Molnár (1909) and Savich (1921) reported that denervated fistulae secreted more juice, especially in response to feeding, than fistulae with their nervous connections intact.

Nasset and his co-workers investigated the output of transplanted fistulae before and after cutting the pedicle (Pierce, Nasset and Murlin, 1935; Schiffrin and Nasset, 1939). In the latter paper they gave figures showing a decrease in the volume of jejunal secretion, both fed and fasting, after cutting the pedicle, but no change in the ileal secretion. They spoke also of differences in enzyme content, but as they estimated the enzymes of the whole juice, including the deposit, such extraneous sources as cells cast off from the mucosa were not excluded. They stated that the pH and the chloride and CO_2 content were unaffected.

It is certain that when the pedicle is allowed to atrophy gradually no noticeable change in the volume or character of the secretion takes place. Presumably the secretory mechanism, like the circulation, has time to become adjusted to new conditions.

Chemical composition of fistula juice. The amount of mucin is very

variable, depending on the amount of floccular material in the juice, and may be so low that only a slight turbidity develops with 2 per cent acetic acid.

The pH is usually given as 8 to 9 (e.g., Bickel and Kanitz (man), 1934; Amberg and Sawyer (dog), 1926) but de Beer *et al.* (1935), with more careful collection and better chemical methods, gave values of 6.30 to 7.28 for the jejunum and 7.61 for the ileum. Schiffrin and Nasset (1939) gave 6.9 to 8.6 for jejunum and ileum. The neutralizing value is variously given as from 0.02 to 0.67 per cent Na_2CO_3.

De Beer *et al.* give extensive tables of their analyses of jejunal, ileal and colonic fistula juice. The outstanding feature of their figures is the relative constancy of the individual kationic concentrations from one secretion to the other, and the constancy of the anionic sum with reciprocal variation of the Cl and HCO_3. Other experiments by the same workers show that an alteration of Cl content of the blood may be reflected in the *succus entericus* but not an increase in blood HCO_3.

For the various non-protein nitrogen fractions see Ikeda (1934), and for separate analyses of fluid and floccular parts of the juice, Amberg and Sawyer (1926).

Nitrogen excretion. The amount of nitrogenous matter secreted in the *succus entericus* was investigated by Mosenthal (1911). By making fistulae of a known proportion of the intestine in six dogs and collecting the secretion while following the total nitrogen intake and output, he deduced that the intestinal secretion might contain up to 35 per cent of the ingested nitrogen. Since the fecal nitrogen was usually only 10 per cent of the ingested nitrogen Mosenthal argued that at least 60 per cent of the nitrogen secreted into the lumen was reabsorbed. Hermann's (1890) closed loop experiment in which, over a period of weeks, mucin, desquamated epithelial cells and fat droplets formed a semi-solid mass, does not prove that in normal circumstances all this material is excreted with the feces. In such a preparation the material is not subjected to the digestive action of pancreatic juice and the physical action of bile, which would probably cause the death and digestion of almost the whole of this desquamated material.

Lipid excretion. Sperry (1926, 1926–27), and Sperry and Bloor (1924) have brought forward evidence that lipids are secreted by the intestine and, in normal dogs, largely reabsorbed, but escape with the feces when bile is excluded. Shapiro *et al.* (1936) by employing deuterium-containing fats, showed that 65 to 70 per cent of the diet fatty acids were absorbed in the absence of bile and that the increase of

fecal fat in the absence of bile was due to the fats secreted into the intestinal lumen.

ENZYMES. Much work on intestinal enzymes was done in the 19th century but, along with some of the more recent work, it left much to be desired for one or more of the following reasons; in particular quantitative comparisons should be treated with great reserve.

1. Chemical methods were primitive and often inadequate; buffers to keep the pH constant were, for instance, not known.

2. No precautions, or inadequate precautions, were taken to prevent bacterial enzymes from acting on the substrate.

3. No distinction was drawn between the properties of the juice which flows from a fistula, which, especially when collected by mechanical stimulation, contains cells, cell débris, and bacteria, and those of the pure secretion of the intestinal epithelium.

It has been abundantly shown that the juice from an intestinal fistula digests starch, cane-sugar, maltose, lactose, poly- and di-peptides and, to a small degree, fat. It also activates trypsinogen (enterokinase). But some of these enzymes are well known to be present in the cells of the intestinal mucosa, and even long centrifuging of the juice cannot eliminate enzymes which have escaped from ruptured cells. Amongst others Cajori (1933), Koskowski (1926) and Pierce *et al.* (1935) showed that whole juice digested more strongly than centrifuged juice, and the last two showed that digestion was still stronger if the juice was macerated with glass beads, which may be supposed to break up the cells in the deposit and liberate their enzymes.

The following paragraphs briefly survey some of the work on intestinal enzymes. Oppenheimer's *Die Fermente* (1925, 1926, 1936) is monumental and should be consulted.

Peptidases.[1] In the intestinal juice of dogs Salaskin (1902), Kutscher and Seemann (1902), Waldschmidt-Leitz and Waldschmidt-Gräser (1927) Cajori (1933) and others described a peptide splitting action. In man, Hamburger and Hekma (1902), Bickel and Kanitz (1934) and Owles (1937–38) found a similar action. Owles, and Pierce *et al.* (1935) preferred not to centrifuge the juice because this reduced the peptidase content. Cajori described a residual activity after centrifuging but found that the peptone was absorbed disproportionately quickly

[1] "Erepsin" was Cohnheim's name for the peptide-splitting ferment of the intestinal mucosa which he discovered in 1901. Several enzymes with distinctive properties, acting on peptides of different structure or complexity, are now separated, so the name becomes obsolete.

to the rate at which it was split by centrifuged juice. The common observation that after several hours of mechanical stimulation the peptidase activity of the secreted juice is reduced is usually explained as due to washing out of the enzyme from the mucosa, but may be due to removal of the desquamated cells from the lumen.

Waldschmidt-Leitz and Waldschmidt-Gräser (1927) and Glaessner and Stauber (1910), using dogs and rabbits respectively, found peptidase in the mucous membrane of gut to which the pancreatic juice had no access. The latter workers found none in the mucosa of a rabbit with diabetes due to destruction of the pancreas, and Foà (1908) found none in the mucosa of a six months old Vella fistula in a dog. Its absence from intestinal secretion could therefore on occasion be due not only to absence of cells but to the absence of peptidase from the cells. Linderstrøm-Lang (1939) after reviewing recent work considered the peptidases to be typical endo-enzymes.

Enterokinase. This enzyme was discovered in the *succus entericus* of dogs by Schepowalnikow (1899), working in Pavlov's laboratory, and in human juice by Hamburger and Hekma (1902), and the relatively few workers who have looked for it since have always found it present, (e.g., Falloise, 1904–05 (dog); Hermann and Ribère, 1931 (man)). It is developed already in early infancy, according to Austin (1909). Schepowalnikow found that the concentration in the mucosa was greatest in the duodenum and decreased downwards, and Pavlov believed that this was true also of the concentration in the juice. In the mucosa it is chiefly in the villi (Falloise, 1905). Ohno (1930) and Waldschmidt-Leitz and Waldschmidt-Gräser (1927) suggested that the lymphocytes which entered the gut might play a part in activating trypsinogen.

Sawitsch (1904) stated that in a loop of intestine deprived of pancreatic juice the secretion of enterokinase gradually failed. Unfortunately the paper is not available for consultation but the report given by Babkin (1928, p. 782) suggests that neither the methods used nor the results obtained justify this conclusion. The same criticism applies to the increase after food which he reported in 1917 (Orbéli and Sawich). Waldschmidt-Leitz and Harteneck (1925) suggested that pancreatic juice was necessary for the liberation of enterokinase from the epithelial cells but the evidence is indirect and not very convincing. Boldyreff (1928) regularly found it in dog fistula juice, and le Breton and Mocoroa (1931) specifically stated that it was present in the juice from a fistula several weeks old. Foà (1908), however, said that at 6 months it had gone from the juice. According to Savich (1921)

the falling off in enzyme concentration occurred only if the sympathetic nerve supply to the gut was intact, not otherwise.

Rasenkow's (1929) suggestion that enterokinase and secretin are identical cannot be considered seriously; his extracts may have contained almost anything. Comparative studies of the enterokinase in cell-free and cellular juice have not been made.

Lipase. Boldyreff (1904) was the first definitely to demonstrate an action of the *succus entericus* in splitting monobutyrin and fat, but recognized that the lipolysis was weak. Bickel and Kanitz (1934) found no lipase in juice from a human ileal fistula and Owles (1937–38) found very little in juice from the jejunal region obtained in man by the Miller-Abbott tube. Roger and Binet (1921) detected lipolytic activity only after the addition of bile to the juice. Reale (1932–1934) was convinced that lipase was not present in solution in fresh juice but that the concentration gradually rose with the progressive autolysis of desquamated cells. The finding of Roger and Binet and of Jansen (1910) and others, that bile increased the lipolytic acitivity of *succus entericus*, may therefore be due to destruction of cells by the bile.

Amylase. Dobroslawin (1870) first reported an amylolytic action in *succus entericus*, and since that time it has been found by almost every worker who has looked for it (see Babkin, 1928, p. 768; and Oppenheimer, 1925, Bd. I, p. 724). Bierry and Frouin (1906) found that removal of the cells by centrifuge and filter removed the amylase, but Cajori (1933) that it was less reduced than other enzymes by centrifuging. Owles (1937–38) found in temporarily isolated loops of human intestine that even if he did not remove the cells from the secretion the starch splitting activity rapidly diminished, apparently because of the washing out of pancreatic amylase. Wohlgemuth's (1910) observation that ligation of both ducts of the pancreas led to a disappearance of amylase from the feces also suggests that the intestine secretes an insignificant amount. Georgiewsky and Andrejew's (1934–35) observation on fistula bearing animals that a high starch diet increased the amylase in fistula juice is difficult to assess. Cajori found that starch was split by the juice and absorbed from the fistula at about the same rate; he found also that the amylase content of the juice was of the same order as that of the blood and other body fluids and suggested that the enzyme in the juice came from the blood.

Invertase. Leube reported an inverting action of *succus entericus* in 1868. Claude Bernard (1873) found activity in both the mucosa and the secretion. Euler and Svanberg (1921) found that the concen-

tration was greatest in the mucosa of the jejunum, less in the duodenum and least in the ileum. Tubby and Manning (1892) found invertase more often in the mucous than in the fluid part of the juice from a human ileal fistula; Bierry (1912) showed that when juice was centrifuged and passed through a Berkefeld filter it lost its inverting power, Koskowski (1926) that clear juice contained less invertase than turbid or cellular juice. Cajori (1933) and Röhmann and Nagano (1903) combined investigation of the juice with absorption experiments and concluded that cane-sugar was largely inverted after absorption, during its passage through the mucosa. Apart from some leucocytes, the only place in the body where invertase is found is the intestinal epithelium, but it seems likely that the enzyme is not secreted from the cells into the juice.

Maltase. Brown and Heron (1879–80) described the splitting of maltose by intestinal extracts and this has been repeatedly confirmed. Shore and Tebb (1892) and Tebb (1894) demonstrated also that maltase was found in a great variety of animal tissues in contrast with the restricted occurrence of invertase and lactase. Osato (1920) found the greatest concentration of maltase in the jejunum, less in the duodenum and still less in the ileum, while the colon had only a slight activity. He also pointed out that carnivores had a relatively high activity throughout the gut but in the herbivores the concentration decreased rapidly and in the ileum was very small.

In the *succus entericus* Röhmann (1887), Mendel (1896) and others reported activity, often slight. Bierry (1912) freed the juice of cells and microörganisms and still found some maltose. Röhmann and Nagano (1903) found that maltose was split by intestinal juice, but not so fast as it was absorbed by the mucosa.

Lactase. Röhmann and Lappe (1895) reported the presence of a lactose splitting enzyme in intestinal mucosal extracts. This was confirmed by Weinland (1899 and 1900), who advanced some evidence that its presence was restricted to young or growing mammals. Plimmer (1906–07) found it consistently present in the mucosa of young mammals of several species and persistent throughout life in many of them, whether or not they were kept on a milk diet. In an extensive series of experiments he found it impossible to restore lactase to the mucosa where it was absent, or to increase the amount present, by feeding large quantities of lactose. Several authors have reported lactase in *succus entericus* (e.g. Röhmann and Nagano, 1903; Savich, 1917), but the amount was always small and there were many negative results. Cajori

(1935), from experiments on centrifuged juice, came to the conclusion that lactase was not secreted.

The juice from Thiry-Vella fistulae did not yield appreciable amounts of *nuclease* (Levene and London, 1929) though gastro-intestinal fistulae gave juice which hydrolysed nucleic acid but not nucleosides (Levene and Dillon, 1930).

It is doubtful whether *arginase* is present in the intestinal mucosa (Oppenheimer, 1936, p. 578); it does not seem to have been looked for in the juice.

Summarizing: The *succus entericus* has significant and varied digestive powers. The amount of digestion by invertase, lactase, peptidase and lipase appears to run parallel with the cell content of the juice, suggesting that these enzymes are derived from cast off epithelial cells rather than from the mucosa by secretion. The probability is that they exert the most important part of their digestive action during absorption; Starling (1911, p. 127) mentioned that such a view was held by many physiologists, and Howell adopted it in his textbook (1926). The position of maltase is less certain. Reports on the relation of amylase to cell content are contradictory, but it has been so consistently found that its presence in the secretion must be considered likely, at any rate in the dog. Enterokinase has been found by all who have looked for it and is probably the most important of the enzymes. Rona and Weber (1927) and O. Kestner (1930) discuss these and other points associated with intestinal secretion.

It should be kept in mind that almost all the work has been done on dogs, and that species differences are possible. Human intestinal juice contained several enzymes after long and vigorous centrifuging (Hermann and Ribère, 1931) and was found by Hamburger and Hekma (1902 and 1904) to be equally active before and after filtration; in spite of the special fallacies of collecting material from human subjects this is a striking and unusual result. Juice from very thoroughly washed loops of human intestine contained small or moderate amounts of invertase, peptidase and lipase (Owles, 1937–38 b); the enzyme activity was said to be reduced by centrifuging, but comparative figures were not given.

THE CONTROL OF SECRETION. *Local stimulation.* The local stimulation of a fistula has already been discussed. Local stimulation has been applied by Owles (1937–38 c) to a loop of intestine in continuity, isolated by balloons. In response to hypertonic solutions there was a considerable output of fluid; air or normal saline had little or no effect on the volume produced.

The influence of extrinsic nerves: The vagus. There appears to be only one series of experiments in which vagal stimulation produced intestinal secretion. Savitch and Soshestvensky (1917) stimulated the vagi in the neck of decapitate cats and found that, after a latent period of 1 to 1½ hours, they could milk juice out of the small intestine at intervals. Their method of squeezing out the juice, producing as it does a local mechanical stimulation, was highly undesirable, but considerable quantities of juice were collected, up to 18 cc. an hour. The long latent period is peculiar, but they explained it by supposing that the vagus carried inhibitory as well as secretory fibers. Ten milligrams of atropine inhibited the secretion for ½ hour. It is of interest that no anesthetics were used in these experiments, since they have been shown (in the duodenum) to inhibit the secretory response to vagal stimulation.

In the experiments of Wright *et al.* (1940) stimulation of the vagus in cats caused secretion from the upper part of the duodenum, the mucoid constituent of the juice so produced coming from the Brunner's gland area. A small amount of watery juice came from the part of the duodenum just distal to the Brunner's glands but in no experiment was secretion elicited from the rest of the small intestine, though the stimulus was always adequate to produce secretion from the stomach and upper duodenum. In two experiments stimulation of the vagus produced a secretion from the intestine after the splanchnics had been cut in the thorax (cutting the splanchnics alone did not cause secretion).

There is thus not enough evidence to enable one to say definitely what influence the vagus has on the secretion of the small intestine. Possibly the right experimental conditions have not yet been devised.

The sympathetic. The influence of the extrinsic nerves on intestinal secretion first received attention in the middle of last century when in 1859 Bernard reported that the removal of the solar ganglion caused fluid to be produced in the intestine. Moreau (1868) investigated further and found that if he cut the mesenteric nerves to a loop of intestine it filled with fluid. This observation was many times repeated (Radziejewski, 1870; Hanau, 1886; Leubuscher and Tecklenburg, 1894; Mendel, 1896; Falloise, 1904; Molnár, 1909; Wright *et al.*, 1940).

A committee of the British Association (Pye-Smith *et al.*, 1874; Brunton and Pye-Smith, 1875 and 1876) performed experiments from which they concluded that the nervous centers which controlled intestinal secretion lay in the small ganglia of the solar and superior mesenteric plexuses, since they found that secretion was unaffected by cutting the splanchnics, the vagi, or the dorso-lumbar part of the cord. Recently the subject was reëxamined (Wright *et al.*) on decerebrate and

decapitate cats, with the following results: cutting the greater splanchnic nerves in the thorax caused secretion from the duodenum but not from any other level of the small intestine; cutting all the preganglionic fibers was followed by secretion from all the small intestine; the ganglia of the solar plexus took on the power to inhibit secretion within a few days after the preganglionic fibers had been cut; this "paralytic" secretion was inhibited by atropine. Mitsuda (1924) thought that the extra-intestinal sympathetic inhibited the secretory glands chiefly through the intramural sympathetic ganglia, but his experiments were inadequate.

The question has been debated whether the "paralytic" fluid so produced does in fact represent a secretion or whether it is a transudate (Kühne, 1878; Vulpian, 1874; Leubuscher and Tecklenburg, 1894; Wertheimer, 1902; Starling, 1911, p. 122). The majority have considered it to be a true secretion, a view strengthened by the observation that the "paralytic" fluid from the Brunner's gland area (see section A) contains the mucin typical of that region.

The juice is usually turbid and yellowish and contains a variable amount of thick gray or white mucus. The specific gravity is reported to be about 1009, the alkalinity equivalent to 0.20 per cent Na_2CO_3; Falloise (1904) gives the soluble and insoluble salts as 0.66 per cent and the organic solids as 0.75 per cent. He tabulates the comparative analyses of fistula juice, paralytic juice and transudate. Of the enzymes tested, invertase, maltase, peptidase (Mendel, 1896; Falloise) and enterokinase (Falloise; Wertheimer) have been reported present, protease and lactase absent (Mendel, Falloise, Tecklenburg, 1894), lipase and amylase contradictory (Falloise, Tecklenburg, Wertheimer, Molnár, 1909). Lately peptidase and invertase were again found, and were attributed to the cells in the juice (Wright *et al.*, 1940).

Mendel and Falloise adduced the presence of ferments in the juice as evidence that it was a true secretion, not realizing that they might have been accidental inclusions. The mucin and bicarbonate content are probably the best indications that it is in fact a secretion. The recent confirmation (Wright *et al.*) of Hanau's suggestion that atropine inhibits the continued formation of the juice is also in favor of its being a true secretion. The suggestion made by Starling (1911, p. 122), that it arises as a result of the vaso-dilatation is unsupported by any evidence.

The influence of drugs. It has long been known that pilocarpine will produce a flow of thin fluid from the intestine (Masloff, 1882; Vella,

1888 a; Hamburger, 1895; Glinski, 1891; Schepowalnikow, 1899; Sawitsch, 1904); as secretion continues the enzyme content of the juice diminishes.

Wright *et al.* found that eserine alone, in sufficient dosage, caused a copious secretion from the whole length of the intestine, without any concurrent flow of tears, saliva or tracheal mucus, or sweating of the pads of the feet. The juice was fluid, yellow, turbid and mixed with a variable amount of white mucus and débris. The administration of acetyl-choline to an eserinized animal often resulted in severe damage to the mucosa with stripping of villi. Atropine inhibited the action of eserine and acetylcholine.

Histamine given subcutaneously was stated by Koskowski (1926) to produce secretion from a Thiry-Vella fistula in dogs, but his evidence is not impressive. Nechoroschew (1929), too, claimed that histamine caused secretion but his description is far from clear and the quantities he obtained were small. Berndt and Ravdin (1934) and Cajori (1933) reported that the secretion of a modified Thiry loop, collected by a Johnston (1932–33) catheter, was considerably augmented by histamine; the increase reached its maximum half an hour after the injection. Lim *et al.* (1922–23) found no effect on the human duodenum. Owles (1937–38) considered that histamine had no specific action on the secretion of *succus entericus* in man.

Certain other drugs are said to produce small intestinal secretion when injected parenterally. Komarow found that large doses of methylguanidin (5–7 mgm./kgm.) (1924 b), carnosin (20–40 mgm./kgm.) (1924 c) and carnitin (1926) caused a secretion from the intestine as well as the salivary, lachrymal, bronchial and gastric glands.

The influence of hormones. The first report of a hormonal stimulus to intestinal secretion came from Delezenne and Frouin (1904). They obtained an abundant flow of juice from lower duodenal and upper jejunal fistulae by the intravenous injection of secretin-containing preparations. Bottazzi and Gabrieli (1905–06) using very large amounts of duodenal and jejunal extracts (15–40 cc.) also obtained a secretion, but Foà (1908), repeating their work, found that the extracts killed many dogs and only occasionally produced juice. Frouin (1905) got a secretion by intravenous injections of the *succus entericus* itself. Brynk (1911) put 200 to 300 cc. of HCl of different strengths into the stomach without increasing the secretion from a Thiry-Vella fistula. Delezenne and Frouin had obtained such an increase, but believed that the stimulus to secretion came from the intestine and not from the

stomach; they showed that secretion could be produced from a lower duodenal or upper jejunal fistula (though not from an ileal fistula) by putting acid into a second high fistula in the same dog. Savich (1921), too, found that HCl in one fistula increased the secretion from another but only if the nerves to the fistulae had been cut; a denervated fistula secreted also in response to feeding; Savich concluded that the nerves inhibited secretion.

Brestkin and Savich (1927) concluded that some humoral mechanism for stimulation existed. Ågren (1934) reported that highly purified secretin caused intestinal secretion but his methods of testing seem rather inadequate and the amounts of secretion produced were very small. Nasset and his collaborators in a series of papers (Nasset, 1938; Nasset *et al.* 1938; Schiffrin and Nasset, 1939) have described a new hormone, distinct from secretin, which they have named "enterocrinin." The hormone was obtained from intestinal mucosa by extracting with HCl and alcohol and precipitating with NaCl, then extracting with glacial acetic acid and precipitating with ether and picric acid. The product when injected intravenously increased the amount of juice which could be obtained from a fistula by a catheter. Whether the methods of assay adopted by Nasset and his collaborators were adequate it is difficult to say; no juice could be collected without concurrent mechanical stimulus and the reason for this, as well as the mechanism of enterocrinin production and action, require further elucidation.

There is thus a varied collection of stimuli which will produce a flow of fluid from the intestine, namely, local mechanical, chemical and electrical stimuli, feeding and the introduction of acid into the stomach and upper intestine, secretin and "enterocrinin," sympathectomy, pilocarpine, eserin, and possibly histamine and other "non specific" substances. Vagal stimulation has not been clearly proved to produce secretion. Atropine inhibits certain of the secretory effects. Except for "periodic" secretion, which it inhibits, the ingestion of food has not been found to have much effect on the secretion of a fistula, though J. W. Boldyreff (1928) stated that bones in the food increased the secretion in dogs. Brestkin and Savich (1927) noted an increased flow of juice from denervated fistulae after food.

One difficulty in evaluating conclusions is that the results from acute experiments and from experiments on fistula-bearing animals are different. Most fistulae are partially denervated, and their final denervation as the pedicle atrophies is a slow process, allowing time for autonomic and hormonal balance to be readjusted; for example, al-

though sympathectomy produces much juice in the acute experiment, whether or not the vagi are cut, there is no hypersecretion from a totally transplanted fistula; similarly, the hypersecretion which follows abdominal sympathectomy disappears in a few days. Clearly knowledge must progress further before a definite view can be expressed as to which are the important factors in the control of intestinal secretion in the intact animal. It is still more difficult to estimate how all the factors involved are integrated and the cloud which covers this aspect of intestinal physiology is perhaps the cause of the fog surrounding many aspects of the pathology of the intestine.

Argentaffine and Paneth cells. It has been mentioned that little is known of the products of these cytologically very easily recognized cells. Efforts have been made, especially with the argentaffine cells, to identify their products by histo-chemical means. The reader is referred to Lison's book (1936) and Jacobson's paper (1939) for a discussion of the argentaffine cells, and to the article by Patzelt (1936) for the Paneth cells.

Functions of the succus entericus. It has been generally accepted that the *succus entericus* has an important part to play in digestion by virtue of the enzymes which it contains. However, from a survey of the literature it is by no means certain that the evidence is strong enough to support this view. It seems likely that few of the enzymes are actually secreted but that most of them enter the juice, as it were incidentally, from the break-up of cells. There can be no doubt that there is a constant and considerable desquamation of epithelial cells into the lumen of the intestine, shown, for instance by the large number of mitotic figures in the crypts of Lieberkühn, and by the presence of cells in the juice from a fistula.

The balance of evidence is that juice obtained by experimental methods from the jejunum and ileum is an alkaline fluid which, like that from the duodenum, contains only two secreted digestive enzymes—amylase and enterokinase. There is no satisfactory evidence whether such experimental juice has the same properties as the *succus entericus* secreted during the passage of chyme along the intestine, but it may be assumed that during digestion a fluid is secreted which has other functions besides that of contributing enzymes. It is well recognized that water and salt solutions are very rapidly taken up from the small intestine, and we have shown in this paper that duodenal secretion itself can be absorbed from the jejunum. It may be necessary for a constant secretion of fluid to take place from the crypts of Lieberkühn to keep the food particles in suspension while they are attacked by the pancreatic enzymes, and as the products of digestion are absorbed, water and salts go with them. One may therefore envisage a circulation of fluid during active digestion, the secretion passing out from the crypts of

Lieberkühn into the intestinal lumen and back into the villi. (Wright *et al.*, 1940.)

C. Cecum and colon. *Anatomy.* The mammalian cecum and colon are lined by a mucous membrane composed of crypts but no villi. The epithelial lining of the crypts is composed of large numbers of goblet cells interspersed between clear protoplasmic cells of various shapes and staining properties. As in the small intestine, the supply of new cells to replace those desquamated at the surface comes from the depths of the crypts where mitoses are frequent.

The cecum and colon are organs in which water absorption, with consequent drying of the feces, appears, at least in carnivora, to be the chief function. In herbivora they are the site of considerable digestion by bacterial action, especially of cellulose.

The secretory activity of these organs has been investigated by chronic fistula and acute experiments.

Secretion by a fistula: The collection of juice. The amount of spontaneous secretion produced by colonic fistulae in dogs is very small, indeed usually nothing escapes, though a gradually increasing plug of inspissated mucus collects inside (Florey, 1930). Babkin states that Berlazki (1903) collected 0.03 cc. an hour from one dog and 0.12 from another.

As in the small intestine, local mechanical stimulation by a tube increased the amount of juice, e.g., from 0.03 cc. to 0.24 cc. an hour in Berlazki's dog. De Beer, Johnston and Wilson (1935) collected as much as 19 cc. in 2 hours. Russian workers (Babkin, 1928, p. 798 to 799) could obtain no evidence that secretion was influenced by feeding and they concluded that it was almost independent of events in the rest of the alimentary canal and depended mainly on local stimulation.

Composition of fistula juice. The material collection from cecal and colonic fistulae varies from clumps of white mucus to an almost watery fluid. Typically it is viscous and opalescent, with mucus unevenly distributed through it. Its reaction is alkaline, equivalent to an 0.04332 (1) per cent solution of Na_2CO_3 (Babkin, 1928, p. 797). De Beer, Johnston and Wilson (1935) found from 85.8 to 93.3 milli-equivalents of HCO_3 per liter, in 3 estimations. The specific gravity was 1,06131 and the fluid part of the secretion contained 98.6 per cent water, 0.63 per cent organic and 0.68 per cent inorganic substances (Babkin, 1928, p. 797).

Vella (1888b) claimed that cecal and colonic juice contained amylase, invertase and a proteolytic enzyme. Klug and Koreck (1883) in re-

porting their own findings—that secretion from the colon of dogs had no digestive action on starch, fibrin or olive oil—confirmed the negative conclusions of Czerny and Latschenberger (1874) and Marckwald (1875). In cecal secretion Strashesko (1904) found amylase, maltase, invertase and peptidase but no lactase or protease. To this list Wakabayashi and Wohlegemuth (1911) added a weak lipase. Enterokinase has not been found by any investigator.

Maestrini (1916) introduced small sacs into the intestine and also studied the juice and mucosal extracts. He concluded that peptidase, amylase and lipase were present in the proximal colon of the dog but that lipase was absent in the sheep; the enzymes decreased towards the lower colon except that invertase was sometimes present throughout the organ.

Roger (1905) has described a coagulating mucinase in fecal matter, Ciaccio (1906) and Riva (1905) confirmed this work, but Harding (1934) was unable to detect the presence of this enzyme.

Control of secretion. Local stimulation. It is a familiar observation in patients that the secretion of mucus accompanies inflammation of the colon. An analysis in cats of the factors involved (Florey, 1930; Florey and Webb, 1931) showed that local stimulation with, for example, dilute mustard oil, caused a considerable secretion of thick mucoid fluid like egg-white. This secretion was not inhibited by atropine or cocaine. The local application of histamine (1/1000), peptone (20 per cent), acetyl-choline (1/1000) or adenosine (1 per cent) was ineffective in causing secretion. Lium (1939) found, however, that 1 per cent acetylcholine applied to colonic "patches" on the abdominal wall produced secretion and gross damage. Acids applied locally, drying, rubbing and heating were also effective local stimulants (Florey, 1930). The conclusion was that the mucus-producing cells (goblets) were directly stimulated by the irritant to discharge their secretion. The irritant appeared to act on some secretory mechanism in the cell which called for oxygen, for in perfusion experiments cyanide inhibited the discharge of goblet cells in response to local irritation (Florey, 1931). It has been noted that goblet cells grown in tissue culture appear to discharge their mucin (Florey, 1930).

The influence of nerves. The nervous control of colonic secretion has been reinvestigated in recent years mainly on acute preparations.

a. *N. erigentes.* (*Pelvic nerves.*) Florey and Drury (1928) in a short note stated that stimulation of the *N. erigentes* in the cat caused secretion from the colon. After further experiments Florey (1930) believed

that there was insufficient evidence for the first conclusion. However, Wright *et al.* (1938) improved the experimental methods considerably and produced consistent results. They showed that prolonged faradic stimulation of the peripheral ends of the cut *N. erigentes* caused a clear mucoid fluid to be secreted by the colon. In contrast with the small amounts reported from fistulae, as much as 55 cc. was collected on one occasion in 8 hours from the distal half of the colon, while in many cats the average rate was about 5 cc. an hour. It appeared that the better the nutritional state of the cat the better was the result. Secretion was closely related to stimulation, stopping when stimulation was stopped and starting again when it was restarted.

Stimulation of the central cut end of one *N. erigens*, the other being intact, caused reflex secretion. The reflex center was in the lumbar cord. Previous reports of reflex excitation of secretion were made by Hay (1883) and by Larson and Bargen (1933b). In the former's experiments the results were not very clear cut and apparently they were never confirmed. Larson and Bargen found that an isolated segment of colon showed an increased secretion at the time of defecation; it was almost certainly, however, deprived of its secretory nerve supply (*N. erigentes*), since the nerves travel along the colon in the muscle coat; the secretion possibly depended on release from sympathetic inhibition. (Larson and Bargen (1933a) reviewed the literature dealing with the distribution of the *N. erigentes* and vagi.)

Additional evidence of the influence of the cholinergic nerves on colonic secretion has been obtained by using drugs. Pilocarpine in large doses has long been known to excite colonic secretion (Heidenhain, 1883; Vella, 1888b; Majewski, 1894; Florey, 1930). Florey suggested that the secretion might be caused by the violence of the muscular action of the colonic wall, but this was subsequently disproved (Wright *et al.*, 1938). Acetyl choline given *sub cutem* to eserinized cats, or applied locally (Lium, 1939), also caused secretion of mucoid juice. Atropine inhibited the secretory action of both nerve stimulation and drugs. Eserine given subcutaneously increased the amount of secretion produced by stimulating the *N. erigentes*. Jones and White (1938) found that pilocarpine and eserine caused secretion when applied to the colonic mucosa of students.

Koskowski (1926) reported that histamine injected intravenously caused a slight colonic secretion.

Anesthetics, especially those of the barbiturate type, were found greatly to reduce or almost to abolish the secretory response (Wright *et al.*, 1938) (cf. section A, duodenal secretion).

b. *Sympathetic nerves.* Section of the sympathetic nerves did not cause a "paralytic" secretion, but if the sympathetic to the colon was stimulated concurrently with the *N. erigentes* the amount of juice was less than when the *N. erigentes* were stimulated alone. The inhibition might be a direct effect on the secreting cells or due to the vascular contraction accompanying sympathetic stimulation.

These observations in the experimental animal show how profoundly the autonomic nerves can affect colonic secretion, and they give some support to the clinical hypothesis that "mucous colitis" or nervous spasmo-myxorrhea can be caused by a derangement of the colonic nervous mechanism. Many writers, e.g., Wakefield and Mayo (1938), Bockus, Bank and Wilkinson (1928), Cawadias (1927), Lium and Porter (1939), even consider that there is clinical evidence of a relationship between the hyper-motility and hyper-secretion of at least one form of mucous colitis and abnormal psychical or emotional states. In this connection, however, certain observers (e.g., Hollander, 1927) have produced evidence that mucous secretion of the colon is excited by the ingestion of food substances to which the patient is allergic.

None of the studies on stimulation of the pre-optic and hypothalamic nuclei have been concerned with colonic secretion, but other pelvic nerve activity (vesical contraction) occurs on stimulating the anterior commissure and adjacent part of the septum, and gastric motility and secretion have been reported following the stimulation of the hypothalamus (Beattie, 1932; Beattie and Sheehan, 1934). The whole question of hypothalamic function is reviewed by Ranson and Magoun (1939).

Characters of the nervous secretion. The secretion described by Wright *et al.* (1938) was a slightly opalescent fluid of varying viscosity. Sometimes it was so viscous that 10 cc. would drop out of a test-tube in one lump, while at other times it was as fluid as water. Small amounts of tough opaque white material could usually be found floating in it. It often had a spermatic odor, an observation also made on fistula secretion (Babkin, 1928, p. 797). It was alkaline, having a pH of 8.3 to 8.4 on collection, rising to 9.1 to 9.2 when CO_2 had been lost. It contained a surprising amount of alkali; 1.0 cc. neutralized 0.4 to 0.6 cc. of N/10 HCl. Other constituents were: organic matter, 0.4 per cent (less in some juices); total inorganic solids, 0.95 per cent; chloride, 0.35 per cent; phosphate, 0.5 mgm. per cent; calcium, 2.0 mgm. per cent. The mucin of the juice was precipitable by acetic acid.

No enterokinase, invertase, polypeptidase, trypsin or lipase were found but dipeptidase and a trace of amylase were present. The

dipeptidase may easily have come from cells in the juice, as no efforts were made to get rid of them. The enzyme content of this acute juice does not correspond very closely with that reported for fistula juice, which is doubtless very rich in cells.

Reabsorption. Permanent fistulae contain a growing plug of inspissated mucus, but in acute experiments the secretion poured out by the colon, even if very mucoid, could apparently be reabsorbed completely, leaving no trace, as soon as the stimulus was withdrawn (Wright *et al.*, 1938). This capacity of the mucosa to absorb its own product re-opens the question whether the same cell that secretes can also absorb.

Histological changes associated with secretion. After secretion, whether produced by drugs, nerve stimulation or by the local application of irritants, the colon of the cat showed a characteristic picture. The cells lining the bottoms of the crypts tended to become flattened, in extreme instances like squames (after mustard oil), and those containing mucus were discharged. The cells on the free surface were relatively much less changed, sometimes even appearing unaffected by the stimulus. This surprising distribution of the exhausted cells, which was regularly seen, has not been explained (Florey, 1930; Florey and Webb, 1931; Florey, 1932; Wright *et al.*, 1938).

The cycle of events in a goblet cell would appear to be as follows. The earliest stage of mucin production, according to Duthie (1933), is associated with the formation of droplets (which can be colored by neutral red) in relation to the basal mitochondria. The droplets migrate into the region of the Golgi body where mucin, stainable by such selective stains as mucicarmine, is elaborated. The view that the Golgi body is involved in the synthesis of mucin was advanced also by Nassonov (1923), Bowen (1924) and Florey (1932). As mucin production continues the droplets compress the nucleus towards the base of the cell and fill out the cell to a "goblet" shape. The mucin may possibly sometimes be discharged in such a way that the cell collapses, producing a "small" cell which can be recognized between the ordinary epithelial cells. In a quick discharge, however (e.g., under the influence of mustard oil), the mucin passes through the free end of the cell, leaving a protoplasmic cell of the usual type with the nucleus not compressed. By careful staining droplets of mucin can usually be demonstrated in the Golgi body area of such cells. If the stimulus is now withdrawn the cell starts to reform mucin, ready to repeat the cycle. The regeneration of mucin by exhausted goblet cells appears to be fairly rapid; a colon

severely depleted of its mucin by mustard oil shows very considerable recovery in two days. A discussion of some of the histological changes, including observations by the older histologists, has been written by Patzelt (1936, p. 111).

The part, if any, which the protoplasmic cells of the colonic mucosa play in secretion is not known, but they certainly lose some of their protoplasm in the secretion accompanying experimental inflammation. It would be interesting to have an analysis of the action of cathartics bearing in mind the points recently brought out about control of colonic secretion.

Excretion. The colon is commonly credited with the ability to excrete calcium, phosphate and heavy metals, but Taylor and Fine (1930), Nicolaysen (1934), Welch *et al.* (1936), and Johnson (1937) found no evidence for the excretion of Ca or PO_4. Cowell (1937), however, concluded from an analysis of the feces that the rabbit colon could excrete calcium, and Cohn and Greenberg (1938), who used rats, showed that 3 per cent of "marked" radio-active phosphorus was excreted by the colon in 8 hours, while 20 per cent to 30 per cent went out in the urine. Youngburg (1937), who examined the intestinal mucosa and contents of rats kept on a phosphorus free diet, concluded that there was a considerable elimination of phosphorus compounds through the intestinal wall. Heupke (1931) could obtain no evidence in a separated colon that aniline dyes, potassium ferrocyanide, quinine or gallic acid were excreted, but traces of iodide, bromide and thiocyanate appeared to be. Bargen *et al.* (1929) reported that parenterally administered neoarsphenamine, mercury, mercurochrome and metaphen were not, and that bismuth sometimes was, passed into the colon, but Leitch (1936–37), reviewing the subject, could find no indisputable evidence for any excretion by the colon of these substances. Boggino (1931) obtained histological evidence that injected iron was excreted by the goblet cells in the guinea pig. Ramond and Popovici (1931) injected iron hyposulphite in rabbits and found that iron was excreted by the cecum and proximal colon; lead, copper, mercury, silver and bismuth also, though very toxic, were excreted in the same region.

Functions of the colonic secretion. Information about the herbivora appears to be very scanty and possibly useful knowledge might be obtained by a study of the comparative physiology of the colonic secretion. The following conclusions as to its possible functions are based for the most part on results from dogs and cats.

It is difficult to believe that the supposed enzymes in the colonic

secretion could perform any important function. For reasons discussed in section B it is doubtful whether any actual secretion of enzymes would be shown if adequate methods and cell free juice were used.

Mucus appears to be the substance of most interest. Even very mucoid juice can be apparently reabsorbed and this means that the colonic mucosa can maintain a layer of mucus on its surface and can vary the thickness by secretion or reabsorption, according to the need of the moment. The mucus no doubt lubricates the solid feces as they pass through the lower colon and also helps to bind together the fecal particles; for example, hydrokollag given by stomach tube to a starved cat was subsequently found rolled into masses in the colon and on section the masses proved to be formed of carbon held together by mucus (Florey, 1933). Probably mucus is not digested in the colon but since a small percentage of colonic mucin makes a very viscous solution, the body loses little protein by mucin secretion even when quite large quantities of "mucus" are voided. Our general ignorance, however, of the fate of mucins in the alimentary tract is profound.

If the feces contain irritant matter fluid is produced, either by the direct local stimulus or *via* nervous reflexes. This protects the mucosa in two ways: first, by diluting the irritant, and secondly, by blocking its diffusion, for which a mucoid fluid is well suited. Evacuation *per anum* may then occur before the mucosa is seriously damaged. We have no exact idea what part nervous control plays in such a process nor how the nervous mechanism comes into play under natural physiological conditions. One can only register astonishment at the quantity of fluid that a cat's colon can produce when its secretory nerves are maximally stimulated.

The alkali content of the secretion is considerable, and probably has the function of neutralizing acids produced by bacterial action. For example, the interior of fecal masses from the cat's colon had a pH as low as 4.8 but at the surface the pH was 7 (Wright *et al.*, 1938). Tisdall and Brown (1924) found that the pH of infant's feces varied between 4.7 and 8.3, but in adults, according to Robinson (1922), it was 7 to 7.5.

To sum up, there is a large body of experimental work on secretion by the intestine, from the pylorus to the rectum, and this work has succeeded in analyzing some of the controlling factors. But we know little of how these factors are integrated to supply normal physiological needs; still less do we know in what way their integration differs from the normal in disease. The chronic preparation adapts itself to interference which in the acute preparation produces marked changes and it is this

adaptability, and the delicate balance of controlling mechanisms, which are so hard to investigate.

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THE SIGNIFICANCE OF HYPERSENSITIVITY IN INFECTIONS

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When a foreign protein, whether bacterial or non-bacterial in origin, enters the tissues by whatever route, there ordinarily occur in the body certain alterations which affect the reactivity of the tissues toward subsequent contact with the protein. When these alterations have occurred, the body is said to have become "hypersensitive" to the foreign protein, for the tissues will then be readily injured by amounts of the protein that are entirely harmless to the normal body. Since all pathogenic microörganisms contain protein, an infected body becomes, as a rule, sensitized to the proteins of the microörganisms which invade the tissues. These proteins may be quite harmless to the normal body, but when the state of hypersensitivity has been established as a result of infection, minute amounts of the proteins will cause local damage and death of tissue, accompanied by acute inflammation; and marked constitutional symptoms and even death will occur when amounts of the proteins which are entirely harmless for the normal body enter the blood stream of the sensitized one. Even bacteria which have little or no power to kill the tissues of the normal body when injected locally, or to produce constitutional symptoms when injected into the blood stream, will exert both of these effects to a marked degree when introduced into the body that has been sensitized by previous contact with the bacteria. Since small numbers of bacteria, or minute amounts of their proteins, suffice to incite hypersensitive reactions in the sensitized body, it is obvious that the hypersensitive state is a factor that has seriously to be reckoned with in the interpretation of the lesions and symptoms of infectious diseases.

DIFFERENCES BETWEEN BACTERIAL HYPERSENSITIVITY AND HYPERSENSITIVITY OF THE ARTHUS TYPE. It is important at the outset to stress the fact that there are certain differences between the protein hypersensitivity that is established as a result of contact of the tissues with microörganisms, and that which results from contact of the tissues

with a free foreign protein. When a free foreign protein, whether bacterial or non-bacterial in origin, is injected into the tissues, there develops a form of hypersensitivity which has, among others, the following characteristics: 1, the local or constitutional reaction produced by subsequent contact with the protein develops rapidly and reaches its height shortly after the test injection; 2, smooth muscle of the sensitized body is thrown into spasmodic contraction on contact with the specific protein; and 3, the hypersensitive state is transferrable passively to a normal body by injecting into it the serum of the hypersensitive one, i.e., sensitizing antibody is present in the blood stream of the hypersensitive body. This is the "anaphylactic" type of sensitivity. If repeated injections of the protein are made into the tissues at intervals, the local reaction increases in intensity with each injection until, finally, instead of eliciting merely a rapidly appearing and evanescent edema and erythema, such as characterize the local reaction of the anaphylactic state established by a single injection of the protein, a large, edematous, hemorrhagic and necrotic reaction will occur. This intensified local anaphylactic sensitivity is known as the "Arthus type" of hypersensitivity. As in the case of the local reaction in simple anaphylactic hypersensitivity, the onset of the Arthus reaction becomes macroscopically apparent promptly after the introduction of the protein into the tissues, and the hypersensitive state is transferrable passively.

While anaphylactic hypersensitivity to the bacterial protein may develop during infection, the characteristic state of protein hypersensitivity produced by contact of the tissues with microörganisms is different from either the simple anaphylactic or the Arthus type of hypersensitivity, and is ordinarily termed "bacterial hypersensitivity," "hypersensitivity of infection," or "tuberculin type hypersensitivity." The necrotizing inflammatory reaction produced by contact of the sensitized tissues with the specific bacteria or their extracted protein may, in its fully developed state, be indistinguishable in appearance from an Arthus reaction, but the state of bacterial hypersensitivity differs from the Arthus type of hypersensitivity in the following ways: 1, the local or constitutional reaction resulting from contact with the bacteria or their proteins develops slowly, requiring some hours before it becomes obvious, in contrast to the prompt appearance of the reaction in the Arthus sensitized body; 2, the smooth muscle of the sensitized body is not thrown into spasmodic contraction by contact with the protein; and 3, the hypersensitive state is not transferable passively.

Exceptions to these and to other differences between bacterial and

Arthus type hypersensitivity may be encountered. For example, occasional, though irregular and unreproducible passive transfer of bacterial hypersensitivity has been recorded (1); and a transitory state of tissue reactivity of the slowly developing type may precede the appearance of anaphylactic sensitivity following the injection of certain foreign proteins into certain animals (2) (3). Exceptions such as these have led some investigators to believe that bacterial and Arthus hypersensitivity are states that are fundamentally the same; and this may, indeed, be the case. Most students of the matter, however, prefer to separate the two states until further information is available, for the above-mentioned differences are striking, and are the rule; and there are additional and no less striking differences. For example, the state of hypersensitivity that results from contact of the tissues with bacteria, whether living or dead, confers upon the body the ability to react hypersensitively not only to the protein derived from the bacteria, but also to the injection of the intact bacteria themselves. In contrast, the body that is brought into the state of Arthus hypersensitivity by the repeated injection of the protein derived from a given bacterium will react violently to the injection of that protein, but will not react hypersensitively to the injection of the intact bacteria (4) (5). We cannot enter here into a discussion of these differences, but it may be remarked that the failure properly to appreciate them has led to considerable confusion, and to certain erroneous conclusions that will be mentioned below.

DIFFERENCES BETWEEN THE MECHANISM OF THE TISSUE DAMAGE IN BACTERIAL AND IN ARTHUS HYPERSENSITIVITY. An important difference between the Arthus and the bacterial type of hypersensitivity lies in the site of sensitivity in the two states. In 1922, Holst (87) made a pioneering attempt to learn whether washed cells from the tuberculous body are hypersensitive to tuberculoprotein *in vitro*, but he stated that he was unable to reach any satisfactory conclusion by the technique employed. Rich and Lewis (6), using the tissue culture technique, were subsequently able to show that when tissue or blood cells of the body with bacterial hypersensitivity are removed from the body and well washed, they are killed *in vitro* by amounts of the bacterial protein which are innocuous to the cells of the normal body under the same conditions. This was confirmed by Aronson (7) and by Moen and Swift (8). Barg (9) and Aronson (13) found that the cells of the Arthus sensitized body, in contrast, can thrive *in vitro* in contact with the

specific protein[1]. Rich, Lewis and Gay (14) have confirmed this observation, and have further shown that the cells from human beings with asthma and hay fever, who were extremely sensitive to the specific antigen, were unharmed by contact with the antigen in tissue cultures. It is, indeed, impressive to see the cells of the Arthus sensitized body growing unharmed in tissue culture while bathed in an amount of the foreign protein that produces a necrotic lesion when injected into the body from which the cells were derived.

This, then, appears to be a fundamental difference between the hypersensitivity of infection and the Arthus type of hypersensitivity: in the former, the local tissue damage produced by the protein is due in large part to sensitization of the individual tissue cells; in the latter there is no generalized tissue hypersensitivity. How, then, is necrosis of tissue produced in the Arthus reaction, if the tissue cells at large are not sensitized?

It was Opie's (15) opinion that the specific precipitate that is formed by the interaction of the foreign protein with the precipitin of the sensitized body is a toxic substance that is responsible, in part at least, for the cellular damage that characterizes the Arthus reaction. The precipitate does appear to attract leucocytes when injected into the tissues of the normal body, but it does not cause necrosis (15); and in the tissue culture studies of Aronson (13) and of Rich, Lewis and Gay (14) the cells of the Arthus sensitized body were unharmed by contact with the specific precipitate. It is therefore unlikely that specific precipitate is directly responsible for the death of extravascular tissues in the Arthus reaction.

It has long been observed that vascular damage is a prominent feature of the histology of the Arthus reaction. Arthus (16), himself, noted the hemorrhagic character of the reaction; Opie (15), Gerlach (17), Laporte (30), Pagel (36) and others have well described histologically the vascular damage and thrombosis at the site of the reaction; and Abell and Schenck (18) observed these effects directly in the living rabbit's ear.

[1] Mendeléeff (11) and Sereni and Garofolini (12) have stated that the cells of the anaphylactic body are damaged *in vitro* by contact with the specific protein. The former writer makes only a very brief statement about the matter, and the experiments of the latter investigators are vitiated by the fact that the cells of the sensitized body failed to migrate and grow in the plasma of the same body even when the foreign protein was not added to the cultures. Neither Holst (87) nor Meyer and Loewenthal (10) could detect any damage caused by contact of the cells of the anaphylactic body with the specific protein *in vitro*.

The prominence of this vascular damage has suggested that the resulting interference with nutrition is an important factor in producing the tissue damage. Extending the above mentioned tissue culture studies to the intact body, Rich and Follis (19) have shown that whereas, in the Arthus sensitized body, tissues that contain blood vessels are readily killed by contact with the specific protein, the cells of a normally avascular tissue (cornea) are not killed by such contact[2]. If, however, the cornea is vascularized before the test injection is made, there occurs an Arthus reaction, with thrombosis of the newly formed capillaries, necrosis of their walls and hemorrhage.

The evidence derived from the *in vitro* and *in vivo* studies, therefore, indicates that in bacterial hypersensitivity there exists a type of sensitization of tissue cells which causes their death on contact with the specific bacterial protein, while in the Arthus type of hypersensitivity no such sensitization of the extra-vascular tissue cells exists. In the latter type of hypersensitivity, however, vascular damage does occur as a result of contact with the specific protein, and the surrounding tissue damage appears to be due to interference with nutrition resulting from the vascular damage and from the clogging of the tissue spaces with hemorrhage and exudate.

IS BACTERIAL HYPERSENSITIVITY DEPENDENT UPON ANTIBODY? There is no doubt that the simple anaphylactic and the Arthus type of hypersensitivity depend upon the presence of antibody. The fact that the injection of the serum of the sensitized body into a normal one confers sensitivity upon the latter is plain enough evidence of this. As was mentioned above, however, bacterial hypersensitivity is not transferable passively, and in this type of hypersensitivity no one has been able convincingly to demonstrate sensitizing antibody by any method. There are, nevertheless, a number of persuasive reasons for believing that bacterial hypersensitivity depends upon specific antibody.

To begin with, the fact that the antibody is not present in the circulation in an amount sufficient to permit passive transfer is no important argument against the view that this type of hypersensitivity is determined by antibody, for it is well known that even in anaphylactic hypersensitivity, in which sensitizing antibody is readily demonstrable in the serum, the increment of antibody that circulates in the blood stream is an excess which is entirely unnecessary for the hypersensitive reaction. The effective antibody is that portion which is intimately associated with the cells, whether actually attached to them or present in the im-

[2] It may be stated that when antibodies are present in the blood they penetrate into the normal cornea (20) (21).

mediately adjacent tissue fluids. Smooth muscle of the sensitized body, washed free of blood, contracts spasmodically on contact with the specific protein; and anaphylactic sensitivity may persist for a time after the circulating antibody has fallen to so low a level as to be indetectable by the usual tests.

One of the most persuasive reasons for believing that antibody is concerned in bacterial hypersensitivity is the high degree of specificity of the phenomenon—a degree of specificity that is difficult to account for on any basis other than as an antigen-antibody reaction. While it is true that cross reactions occur in certain instances (22) (due, presumably, to chemical similarities between the proteins of two different types of bacteria), these are exceptions; and the local reactions produced by the heterologous bacteria are usually less strong and frequently less typical than those produced by the bacterium that was responsible for the establishment of sensitivity. These cross reactions do not in the least disturb the probability that the state of hypersensitivity is dependent upon a specific antibody, for cross reactions are familiar even in the case of serological reactions that are definitely dependent upon specific antibody (cf. the agglutination of the proteus bacillus by the serum of patients infected with the Rickettsia of typhus fever).

In further support of the view that antibody is concerned in bacterial hypersensitivity it is pertinent to mention the anamnestic reaction. When a specific antibody has once been produced as a result of contact of the tissues with a foreign protein, the amount of antibody in the circulation gradually decreases with the passing of time until, at length, none at all can be detected. If, now, the antigen enters the tissues again, the specific antibody will reappear in the circulation, and will reach a given level in a much shorter time than was required following the first contact with the antigen. It was recognized long ago by von Pirquet (23) that when anaphylactic hypersensitivity, which depends upon antibody, has once been established it will gradually wane if there is no further contact with the antigen; but when the hypersensitivity has waned, the re-introduction of the antigen into the body causes the specific antibody and the hypersensitive state to appear again in a decidedly shorter time than was required following the first contact of the body with the antigen. It is significant, therefore, that this anamnestic reaction, which is so characteristic of antibody formation, is also a characteristic of bacterial hypersensitivity. Bacterial hypersensitivity declines to a very low level with the passing of time after an infection, but a fresh contact of the tissues with the specific bacteria will

cause the hypersensitivity to return to a high level in a shorter time than was required for its establishment following the first infection (24) (25). This will be discussed further, below.

Additional evidence that bacterial hypersensitivity is dependent upon antibody is contained in the phenomenon of specific desensitization. In the forms of hypersensitivity (anaphylactic, Arthus) known to be dependent upon antibody, the state of sensitivity can be greatly depressed or abolished by the proper injection of the specific protein to which the body is sensitized. It is significant, therefore, that bacterial hypersensitivity likewise can be depressed or abolished by desensitization, and that the same desensitizing procedures that are most effective toward this end in the case of the hypersensitive states known to be dependent upon antibody are precisely those that are most effective in ridding the body of bacterial hypersensitivity.

These considerations (and others could be cited) may suffice to show that there are close parallelisms between bacterial hypersensitivity and the forms of hypersensitivity known to be dependent upon antibody. It seems altogether probable that bacterial hypersensitivity is likewise dependent upon antibody, but that the antibody is closely bound to the cells, as indicated by the above cited *in vitro* experiments, and that too little excess antibody accumulates in the circulation to permit passive transfer.

THE NATURE OF THE SENSITIZING ANTIGEN. In the overwhelming majority of the instances in which anaphylactic hypersensitivity has been observed, the sensitizing antigen has been a protein. Attempts to induce hypersensitivity to substances other than proteins have so rarely been successful that it is clear that the animal body has little tendency to become sensitized as a result of contact with most non-protein substances. Indeed, the infrequency of sensitization to non-protein substances, and the almost universal sensitizing capacity of proteins, has led to the suspicion that proteins may be implicated in some way even in the instances of sensitization to non-protein substances. This possibility has been strengthened through the work of Landsteiner (37) and others, who have shown that non-protein substances of many types which are not antigenic in themselves may become so if they are united to proteins. The addition of such substances to a protein molecule can alter the antigenic specificity of the protein in such a way that when hypersensitivity is produced by the new molecule the body will be specifically sensitized to the non-protein component of the molecule, and will react hypersensitively to that component in

its native state, unattached to protein. It has been shown by Klopstock and Selter (38) and others that a non-protein, non-antigenic substance may unite with the protein of the blood if simply mixed with the serum *in vitro*, and that the new molecule has then the power to incite the formation of antibody specific for the non-protein component. It is altogether possible, therefore, that when the body becomes sensitized to non-protein substances such as carbohydrates or drugs, the sensitization may result from the union of a non-antigenic substance with the plasma or tissue protein, thus forming a new and "foreign" protein molecule. The matter is certainly not a settled one, however, and it must at present remain a question whether non-protein substances that induce antibody formation must attach themselves to body protein before becoming antigenic.

In the body sensitized by infection, it is only the protein fraction of the bacteria that is capable of eliciting the tuberculin type of reaction characteristic of bacterial hypersensitivity. The body may become sensitized to the bacterial polysaccharide during infection, but the hypersensitivity to the polysaccharide is always of the anaphylactic type (39) (40) (86). It is important to note that while the extracted bacterial proteins are active in eliciting tuberculin type reactions in the body sensitized by infection, the injection of these proteins into the normal body has so far produced only the anaphylactic or Arthus type of sensitization. It is true that there are reports claiming the establishment of the bacterial type of sensitivity as a result of the injection of bacterial proteins, but those reports are not accompanied by evidence that the hypersensitivity so produced actually satisfied the criteria of bacterial hypersensitivity. Whenever proper study of the type of hypersensitivity produced by the injection of bacterial proteins has been made, the sensitivity has been found to conform to the anaphylactic or Arthus rather than to the bacterial type (41) (42). It appears, therefore, that the true sensitizing antigen responsible for bacterial hypersensitivity has not yet been isolated from any bacterium. The very interesting work of Dienes and Simon (2), Simon (142) and others (3) has shown that a transient state of cutaneous reactivity resembling bacterial hypersensitivity may appear several days after the injection of certain non-bacterial proteins into certain animals, disappearing as anaphylactic sensitivity appears. If that state proves to be actually identical with bacterial hypersensitivity, and if it can be produced by extracted bacterial proteins, those proteins would have to be regarded as representing, perhaps, the true sensitizing antigens of bacterial

hypersensitivity; but if that be so, then some additional factor associated with bacteria must be necessary for the persistence of the sensitivity that results from contact of the tissue with either living or dead bacteria.

THE RÔLE OF HYPERSENSITIVITY IN THE PRODUCTION OF SYMPTOMS. The symptoms that occur during infections may be caused by a variety of factors (toxic products derived from the microörganisms and from necrotic tissue; disturbance of the function of various organs; hypersensitivity) and it is frequently impossible to determine just to what degree each separate factor is responsible for the observed symptoms. This is rendered particularly difficult by the fact that each of several different factors may have the power to cause a single given symptom. Fever, for example, can be caused by toxic bacterial products, by products of necrotic tissue, by hypersensitivity to bland bacterial products. It is therefore difficult to separate the effects of hypersensitivity from those caused by other factors occurring during the progress of natural infection. It is, nevertheless, clear that the hypersensitive state can be responsible for a large part of the symptomatology that characterizes infections. This is brought out with particular clarity in the symptoms that occur during hypersensitive reactions to bland, non-bacterial foreign proteins which do not produce symptoms in the non-hypersensitive body. Serum sickness provides a familiar example of this. Horse serum injected into a normal human being produces no appreciable symptoms, but after the lapse of a week or so, when the body has developed antibodies to the proteins of the foreign serum and is thereby rendered hypersensitive, if some of the serum still remains in the circulation, or if a further injection of serum be made, there will occur symptoms which are strikingly like those common to many infections (fever, malaise, aching joints and back, headache, anorexia, prostration). Likewise, when the body has been sensitized to bacterial proteins as a result of infection, the subsequent injection of small amounts of those proteins after recovery from the infection will cause symptoms of the above-mentioned character. As a familiar example, the proteins of the tubercle bacillus can be injected into the normal body with no resulting symptoms, but if hypersensitivity has been established by a slight and completely resisted tuberculous infection, the injection of minute amounts of the bacillary proteins will produce the train of symptoms described above. Likewise, in the animal sensitized by infection, high fever and severe constitutional symptoms will follow the injection of a number of intact tubercle bacilli which will cause no appreciable

symptoms in the normal animal. Furthermore, if the hypersensitive state be abolished by appropriate desensitizing procedures, amounts of tubercle bacilli or their extracted protein which would cause violent symptoms and death in the hypersensitive body can be injected with impunity. This is familiar in the therapeutic desensitization of tuberculous patients (135) (140), and can be readily demonstrated in experimental animals (26) (27). Years ago von Pirquet (23) drew attention to the analogy between the incubation period of infectious diseases and the latent period that extends from the time of injection of a foreign protein to the appearance of serum sickness. Since, in the latter case, the latent period is known to be the period during which hypersensitivity is becoming established, and the appearance of symptoms to be due to the action of the protein on the hypersensitive body, von Pirquet advanced the reasonable idea that in certain infections the symptoms that appear following the incubation period are due to the development of hypersensitivity. There are many reasons for believing that this is often the case, though in some instances the incubation period may be due, in part at least, to the time required for the invading microörganisms to multiply to numbers sufficient to damage the body by their own toxic products. However, since it is well established that relatively bland bacterial proteins are able to produce in the sensitized body symptoms that are quite like those resulting from the action of true bacterial toxins, it is clear that the hypersensitive state confers a potent toxic activity upon even natively harmless products of bacterial disintegration.

THE INFLUENCE OF HYPERSENSITIVITY ON THE CHARACTER OF THE LESIONS. The hypersensitive state intensifies the degree of inflammation and the amount of tissue necrosis that a given number of bacteria or a given amount of their proteins will produce. These effects of hypersensitivity form the basis of numerous diagnostic tests, such as the tuberculin reaction, and are demonstrable in most acute and chronic infections produced by infecting agents of such widely different types as pyogenic bacteria, fungi, mycobacteria, spirochetes, Rickettsia and filterable viruses. In many cases it is impossible merely by looking at a lesion under the microscope to differentiate the effects of hypersensitivity from those of toxic products elaborated by the microörganisms, but in controlled experiment the influence of hypersensitivity in exaggerating inflammation and necrosis can be revealed in a striking way by comparing the lesions produced by a measured number of bacteria in the normal and in the hypersensitive body. Furthermore, if an estab-

lished hypersensitivity be reduced or abolished by desensitization, the amount of inflammation and necrosis that a given number of bacteria will produce in that body will be correspondingly lessened. This has been clearly demonstrated in both acute (28) and chronic (26) infections. As in the case of the influence of hypersensitivity on symptoms, it may be said that the hypersensitive state converts, in effect, even bland proteins of microörganisms into substances that are highly potent in their power to damage tissue.

It has been the experience of most investigators that except for the presence of eosinophils in some anaphylactic inflammations, there is nothing qualitatively specific about the inflammatory exudate produced by bacteria or their products in the hypersensitive body. Practically all who have studied the evolution of the hypersensitive inflammation have recorded the appearance of an exudate in the initial stage of which polymorphonuclear leucocytes predominate, with a gradual increase in mononuclear cells until, after a day or two, the latter cells are present in abundance (43) (44). This is the usual sequence in acute inflammation produced by irritants in the normal body. Dienes and Mallory (29), however, state that in their experience the inflammatory reaction of bacterial hypersensitivity differs from ordinary acute inflammation and from that of the Arthus reaction in that mononuclear cells predominate in the exudate at all stages from the very beginning; and that even in cutaneous tuberculin reactions involving necrosis of the epithelium polymorphonuclears are rarely predominant. Laporte (30), from a cytological study of tuberculin reactions, states that in weak reactions mononuclears predominate at all stages, but none of his descriptions of early reactions show that to have been the case, and in his conclusion he states that "one finds in all cases an afflux of polymorphonuclears in the first hours and a macrophage reaction later." Dr. R. H. Follis, Jr. (100), in this laboratory, has carefully repeated the technique of Dienes and Mallory and has been unable to confirm their report that mononuclear cells predominate from the beginning in mild and moderately intense tuberculin reactions.

Whether or not mononuclear cells ever actually predominate over polymorphonuclears in the initial stage of the reactions of bacterial hypersensitivity, it is true that mononuclears are often more abundant in early reactions of this type than in the early stages of acute inflammation produced in the normal body by irritants in general. To what extent this may be attributable to hypersensitivity requires further study. In tuberculosis it may be due, in part at least, to the well known

fact that the circulating monocytes are increased during infection, providing a correspondingly greater opportunity for more of these cells to escape at inflamed sites. It may be remarked that Dienes and Mallory (29), Laporte (30) and Lurie (45) noted that more mononuclear cells were present in inflammations produced by non-specific irritants in the tuberculous body than in the normal one. It should be possible to determine *in vitro* whether the mononuclear cells of the hypersensitive body are attracted by tuberculoprotein more strongly than are polymorphonuclear cells, and also, by means of tissue cultures, whether the multiplication of the mononuclears of the hypersensitive body is stimulated by amounts of tuberculoprotein too small to kill the cells.

Some writers (31) (32) state that hypersensitivity is responsible for the development of tubercles and tuberculoid tissue in tuberculosis and in the other infections in which tubercles occur. Others (33) (34), in direct contrast, insist that tubercles are necessary for the development of hypersensitivity. Neither of these generalizations can be accepted without reserve at present. It is true that, in response to a given number of bacilli, tubercle formation occurs more rapidly and more abundantly in the tuberculous body than in the normal one; but the view that the specific tuberculous lesions "represent the response of allergic tissue to the presence of bacteria, and are not produced by special properties of the tubercle bacillus" (32) is not tenable, for tubercles and tuberculoid tissue can be produced in the normal body by the injection of even the lipide extracted from the bacilli (35). Also, tubercles and tuberculoid tissue continue to develop in the tuberculous body that is kept desensitized to the degree that very large amounts of bacilli or tuberculoprotein fail to produce hypersensitive inflammation or necrosis (26) (27). Furthermore, in infections caused by many other types of bacteria the presence of hypersensitivity does not cause the formation of tubercles. As for the view that in tuberculosis "either the actual tubercle or a special type of tissue cellular reaction is essential for the establishment of the state of hypersensitivity" (34) this, too, seems unlikely, for bacterial hypersensitivity develops in the many other infections in which such lesions do not occur at all.

As stated above, the agglomeration of mononuclear phagocytes to form tubercles occurs more rapidly in the tuberculous than in the normal body at sites where bacilli lodge, and this has widely been regarded as a manifestation of specific hypersensitivity. There is, however, a lack of conclusive evidence for that interpretation; and while it may be

correct, certain circumstances relating to hypersensitive reactions warrant a critical attitude toward accelerated tubercle formation until its mechanism is clearly established. The existing evidence informs us that it is the bacillary protein which evokes the specific hypersensitive reaction, while only the lipide derivatives of the bacillus incite tubercle formation. Tubercles are not formed in hypersensitive reactions to tuberculoprotein, except non-specifically at sites where unabsorbed necrotic tissue (especially necrotic fat tissue) acts as a foreign body; and there is no convincing evidence that the tubercle-inciting lipides of the bacillus evoke specific hypersensitive reactions. Holley (129) noted that tuberculous animals responded to the injection of tuberculophosphatide with more inflammation than did normal animals, but he attributed that to the presence of tuberculoprotein in the preparation. Smithburn and Sabin (130) observed that a preparation of tuberculophosphatide (protein free?) caused larger reactions in the tuberculous than in the normal body. The microscopic difference between the reaction in the tuberculous and the normal body consisted, however, not in an acceleration or increase in tubercle formation, but in an increase in inflammatory exudate in which polymorphonuclear leucocytes were prominent. Smithburn and Sabin suggested that the reaction in the tuberculous body might be non-specific and similar to the intensified reaction that various non-specific irritants produce in the tuberculous body. Boquet and Nègre (131) were unable to produce hypersensitive reactions in the tuberculous body by the injection of tuberculophosphatide; and Long (132) was unable to detect any hypersensitive effect when various lipides of the bacillus were injected even into the highly sensitive testis of the hypersensitive body. Since the lipides constitute the only portion of the tubercle bacillus which has been found to stimulate tubercle formation, the more rapid development of epithelioid cells and tubercles in the immune, hypersensitive body may, as suggested by the observations of Lewandowski (123), Lurie (124) and others, be due to the immunity rather than to hypersensitivity, for acquired immunity confers upon the body an enhanced ability to destroy the bacilli and thereby to liberate their tubercle-provoking lipides. These considerations render desirable further studies of the mechanism of accelerated tubercle formation.

Many writers have sought to attribute to hypersensitivity a wide variety of lesions of as yet undetermined pathogenesis (e.g., nephritis, the lesions of rheumatic fever, lobar pneumonia). The arguments so far brought forward have been, at best, only suggestive, and they

cannot be analyzed fairly in the space of the present review. Continued investigation of the rôle of hypersensitivity in such conditions is altogether desirable.

THE RÔLE OF HYPERSENSITIVITY IN RESISTANCE TO INFECTION. During infection with most microörganisms the body usually develops an increased ability to protect itself against the attack of the specific invader. Aside from the production of antitoxins which serve to neutralize toxic products liberated from the bacteria, this acquired resistance manifests itself in two prime ways. First, the spread of bacteria from the place where they lodge is inhibited; and second, their multiplication is restricted, and many or all of them are destroyed. By the side of this acquired resistance to infection there develops the "bacterial type" of hypersensitivity to the protein of the microörganisms. In the early part of this century, the dramatic manifestations of hypersensitivity observed in the experimental animal that had developed acquired resistance during tuberculous infection, together with the failure of attempts to demonstrate the protective activity of the antibodies formed during tuberculous infection, led to the widespread belief that the hypersensitive state is responsible for the increased resistance acquired during infection; and the view that hypersensitivity is an essential or highly important mechanism of immunity was then extended to include infections in general. While the destructive effects of hypersensitive reactions have always been clearly recognized, they have been regarded as necessary evils which had to be borne for the protection of the body as a whole, for it has been assumed that the inhibition of spread of bacteria in the immunized body is accomplished by the mechanical, walling-off effects of the accelerated and exaggerated hypersensitive inflammation, and the exaggerated inflammation has also been assumed to be necessary for the more efficient destruction of bacteria which occurs in the immune body. In the space remaining in the present review it is impossible to treat thoroughly the question of the relation of hypersensitivity to immunity. The reader may consult Pagel's (36) recent review, and the discussions in the papers cited in the present article, and should certainly read the reviews of Opie (46) (48), who has favored the view that hypersensitivity is an important mechanism of immunity. In evaluating any writings on this subject it is advisable that the reader be on his guard against being misled by the lamentable and confusing lack of precision in the use of the terms relating to hypersensitivity and immunity. "Allergy," for example, is used by many only as a synonym for hypersensitivity; others

include in the term all manifestations of acquired immunity; and still others in a single paper, shift their meaning of "allergy" backward and forward from one to the other of the above concepts. On the other hand, the term "immunity" is often applied to states in which the body has been rendered susceptible to injury by a foreign substance rather than protected against it. For example, when hypersensitivity has been established to a bland foreign protein which has no power to injure the normal body, it is common that the investigator speaks of having "immunized" the body against the protein, though in reality he has rendered the body hypersusceptible to injury by it. In discussions of the problem whether increased susceptibility of the tissues to injury by a bacterium (hypersensitivity) is necessary for the protection of the body against that bacterium (immunity), ambiguities of the above-mentioned nature frequently confuse the issue completely.

The view that hypersensitivity is a necessary or highly important mechanism of acquired resistance has been based chiefly upon two premises: 1, that an accelerated and exaggerated hypersensitive inflammation inhibits the spread of bacteria (49) (51); and 2, that hypersensitivity and acquired resistance parallel each other (50). In no case has it been proved that acquired resistance cannot function as well in the absence of hypersensitivity as in its presence. Let us proceed to examine the two above-mentioned generalizations.

Is hypersensitive inflammation the mechanism responsible for the inhibition of spread of bacteria in the immune body? There is no doubt that after inflammation has become well established the exudate can form a mechanical barrier that will inhibit the further spread of bacteria; and it is also well known that the multiplication, survival and spread of certain bacteria are markedly inhibited if they are deposited in tissues or serous cavities which have been inflamed for some time before the bacteria are introduced (52) (53) (54) (49). This fact has led to the assumption that the rapidly developing hypersensitive inflammation is a mechanism responsible mechanically for the inhibition of spread of bacteria in the immune body (48) (51). It is important, however, to point out two facts. First, not all types of bacteria to which the body develops effective acquired immunity are restrained by an area of prepared acute inflammation (53) (55) (56); and second, and more important, even those types of bacteria which are restrained when deposited in a previously inflamed area are not restrained if they are introduced into the normal tissues of the non-immune body under conditions which ensure the development of a rapid hypersensitive

inflammation about them. A number of years ago the writer (58) pointed out that the experiment of depositing bacteria in an area that is inflamed *before* the bacteria are introduced does not at all reproduce what occurs in a hypersensitive reaction. In the latter case the bacteria are deposited in normal tissues, and the hypersensitive inflammation develops about them *after* they reach the tissues. Since bacteria are known to spread with great rapidity from the site where they lodge in the non-immune body (57), and since in the early stages of inflammation particulate matter tends to be spread from the site by the increased movement of tissue fluid, it seemed altogether unlikely that even in the hypersensitive body inflammation could develop rapidly enough in sufficient amount to account mechanically for the remarkable inhibition of spread of bacteria observed in the immunized body; and, indeed, the writer showed that when the time conditions obtaining in the hypersensitive reaction were reproduced by causing an accelerated hypersensitive inflammation to develop about bacteria in the previously normal tissues of a body not immune to the bacteria, the spread of the bacteria was accelerated rather than retarded, and in no case did the rapidly developing hypersensitive inflammation protect the animal from a fatal outcome, even though only minute numbers of bacteria were introduced (58). This result has been confirmed by other investigators, using a variety of different bacteria (59) (60) (61). In subsequent studies the writer (62) (63) showed that in the immunized body the immediate immobilization of bacteria is accomplished by the action of antibody, which causes the bacteria to adhere to each other and to the tissues where they lodge; and the immobilization was shown in passive transfer experiments to be altogether as effective in the complete absence of hypersensitivity as in its presence. This immobilizing action of antibody was confirmed by Cannon and Pacheco (64), Catron (65), Pickrell (153) and Wood (154); and Rich and McKee (66) demonstrated that it operates even in the complete absence of a cellular or fibrinous inflammatory exudate. These considerations render untenable the view that the inhibition of spread of bacteria in the immune body is dependent upon the mechanical walling-off effects of a rapid hypersensitive inflammation.

For many years the relation of bacterial hypersensitivity to immunity was studied almost exclusively in experimental tuberculosis in the guinea pig, and the view that hypersensitivity is necessary for the successful operation of acquired immunity has been based largely upon the fact that in that situation hypersensitivity and acquired resistance

usually parallel each other (50). Occasional clinical (67) (68) and experimental (69) (70) (71) investigators raised their voices against the doctrine that hypersensitivity and immunity necessarily parallel each other, and a number of years ago Rich and McCordock (72) assembled the then existing evidence against the view that hypersensitivity is necessary for immunity. Since then, numerous and weighty observations have been brought forward, demonstrating that acquired resistance does not depend upon hypersensitivity. This latter evidence will be briefly summarized.

The separation of immunity from hypersensitivity by passive transfer. Rich and Brown (73) showed that if the serum of animals that are hypersensitive and immune to the pneumococcus be injected intravenously into normal animals, the immunity will be transferred but not the hypersensitivity. In contrast to the large, necrotic hypersensitive reactions produced in the skin by a given number of pneumococci in the immune, hypersensitive animals, it was ordinarily almost impossible to see any lesion whatever macroscopically in the passively immunized animals, and microscopically only a trivial inflammation (far less at all stages than in either the normal or the hypersensitive animals) was found (63); and yet in the complete absence of a hypersensitive reaction the bacteria were sharply localized at the site, septicemia was prevented, and the animals rapidly overcame the infection which was invariably fatal to non-immune controls. Spared from the destructive effects of hypersensitivity, the passively immunized animals, by all tests, prevented the spread of the pneumococci and resisted the infection altogether as well as did the actively immunized hypersensitive ones, even when subjected to several million times the lethal dose for a non-immune animal.

The lack of parallelism between immunity and hypersensitivity. As stated above, the parallel development of immunity and hypersensitivity has provided one of the main arguments in favor of the belief that immunity is dependent upon hypersensitivity. The parallel development of these two states was, however, studied in practically no infection other than tuberculosis. From this limited information, and without further investigation, the parallelism of immunity and hypersensitivity was assumed to apply as a generalization that covers all infections. There is now convincing evidence that no such generalization is warranted. Even in infections in which hypersensitivity and immunity tend to develop concomitantly, their dissociation has been effected by appropriate methods of immunization. Thus, Swift and Derick (74) have shown that whereas animals immunized by the

injection of *non-hemolytic streptococci* into their tissues develop a high degree of hypersensitivity, the test reactions in animals immunized by repeated intravenous injections are less conspicuous even than those occurring in normal animals. This was confirmed by Clawson (75). Petroff, Branch and Jennings (101), Branch and Cuff (102) and Clawson (103) using the *tubercle bacillus*, found that intravenous immunization often failed to establish hypersensitivity. It is probable that the absence of hypersensitivity in these experiments was due, in part at least, to the desensitizing effect of the repeated intravenous injections of bacteria. In these studies, when resistance to infection was tested it was found to be as high in the immunized, non-hypersensitive animals as in hypersensitive ones. In the case of a chronic infection such as tuberculosis, it might be argued that even though the immunized animals had no hypersensitivity when they received the virulent test infection, hypersensitivity could have appeared after some days as a result of the infection itself. To this it need only be remarked that hypersensitivity can be demonstrated within a few days after the infection of a normal, non-immunized animal, and in this case the hypersensitivity does not prevent the devastating progress of the infection. Some years ago Rich and McCordock (72) presented evidence against the then current belief that immunity and hypersensitivity necessarily parallel each other in tuberculosis; and recently, Freund and Opie (104), from a careful study of the development of these two states in experimental tuberculosis, have concluded that "there is no correlation between intensity of sensitization . . . and resistance to infection." Indeed, Sewall, de Savitsch and Butler (105) concluded from their experiments on acquired immunity in tuberculosis that "*immunity is somewhat inversely proportional to the intensity of local allergic reactions*" (author's italics), and Boquet (127) likewise found that "the intensity of the local reaction diminishes as the immunity engendered by the first infection increases." Below we shall see that even when hypersensitivity is prevented from making its appearance during the course of the experiment following infection, immunized animals are protected against the tubercle bacillus in as high a degree as are hypersensitive ones.

While hypersensitivity has often been observed to appear during *pneumococcal* infection, Mackenzie (76) found that when guinea pigs were immunized by the intraperitoneal route they developed a high degree of acquired immunity, but they did not react hypersensitively to the intracutaneous injection of pneumococci or pneumococcal protein.

Numerous writers, without advancing evidence, have stated that

acquired immunity in *syphilis* is dependent upon a local hypersensitive reaction at the site of reinfection (77) (78) (33). Rich, Chesney and Turner (80) have made a detailed study of this point. Rabbits were immunized by intratesticular inoculation, and at various intervals following the immunizing inoculation they were reinfected intracutaneously with large numbers of virulent spirochetes. The sites of reinfection were carefully observed macroscopically, and were removed at daily and weekly intervals for microscopic study. Although the reinfecting dose of spirochetes was far larger than could ever occur in natural infection, in no case was there the slightest indication of a more prompt or exaggerated inflammatory reaction in the immunized animals as compared with control animals, and yet the former exhibited the characteristic high degree of immunity to the test infection.

The lack of parallelism between hypersensitivity and immunity has been noted in *hemolytic streptococcal* infection by Angevine (83), who found that the intracutaneous injection of an avirulent streptococcus produced a high degree of hypersensitivity with scant immunity, whereas a virulent strain produced the reverse effects. He concluded that "increase in virulence of hemolytic streptococci enhances the ability to protect against local infection and increases antibody formation, but diminishes the production of sensitization."

The lack of parallelism between immunity and hypersensitivity has been observed not only in bacterial and spirochetal infection, but also in *filterable virus infection*. In a study of immunity to the filterable virus of infectious myxomatosis McKee (143) found that hypersensitivity appeared in 7 to 14 days after a single vaccinating injection of killed virus, whereas acquired resistance developed later, and usually only after repeated immunizing injections. In animals infected during the period when sensitization was present without appreciable immunity, the local reaction was more severe and generalized lesions appeared earlier than even in normal controls.

Finally, in this connection, it may be pointed out that all investigators are agreed that there is no parallelism between the degree of hypersensitivity and the titer of protective antibodies. Indeed, in the instances studied so far, the antigen that stimulates the development of protective antibody has been found to be different from that to which the body develops bacterial hypersensitivity. Thus, in the case of the pneumococcus, whereas it is the protein portion of the bacterium to which bacterial hypersensitivity develops (39), it is the polysaccharide fraction that is the potent immunizing antigen (84) (85). Injection

of the purified polysaccharide produces a high degree of immunity against infection with the pneumococcus, and this immunity is not accompanied by the bacterial type of hypersensitivity. Anaphylactic sensitivity to the polysaccharides of bacteria may develop, but there is no evidence whatever that anaphylactic sensitivity plays any rôle in protection against infection. Animals immunized against the tubercle bacillus, for example, ordinarily do not respond with a prompt anaphylactic wheal and erythema to the intracutaneous injection of tubercle bacilli. As in the case of the pneumococcus, it is the polysaccharide that is the effective immunizing antigen of the Friedländer bacillus. Morris (86) passively immunized animals to the Friedländer bacillus with an immune serum that also rendered them anaphylactic to the polysaccharide of the bacillus. He then abolished the anaphylactic state by desensitizing injections of the polysaccharide, and demonstrated that immunity to infection remained intact in the absence of anaphylactic sensitivity. Since the animals were passively immunized they also lacked the bacterial type of hypersensitivity.

These various experiments (and many others could be cited) serve to illustrate the fact that not only is there no necessary parallelism between the degree of immunity and hypersensitivity, but also that acquired immunity can be established in widely different types of infections without the concomitant development of hypersensitivity, and that the immunity thus established is as effective in overcoming the infection as is immunity accompanied by hypersensitivity. Indeed, this situation has the decided advantage that the tissues are spared from the damaging effects of hypersensitivity while the microörganisms are being subjected to the forces of acquired immunity.

The separation of immunity from hypersensitivity by desensitization. The above studies on widely different types of infection made it clear that there is no necessary parallelism between hypersensitivity and immunity, and that acquired immunity can operate efficiently even in the complete absence of hypersensitive inflammation. Willis (24), Sewall, de Savitsch and Butler (105), Calmette (71) and others have shown that the hypersensitivity of immunized animals wanes with the passing of time, but immunity to infection remains intact. Since hypersensitivity appears again during the test infection in such experiments, and since hypersensitivity has often been assumed to be necessary for the operation of immunity in the stage of immunization in which it (hypersensitivity) is present in high degree, it remained to determine whether, in the immunized body in which bacterial hyper-

sensitivity is present in high degree, the maintained abolition of the hypersensitivity by desensitization would leave the immunity unimpaired. In order readily to ensure the persistence of the state of desensitization throughout the entire period of the test infection, Rich, Jennings and Downing (28) first chose acute infections (type I pneumococcus; Pasteurella aviseptica) which regularly run their course to a fatal termination in non-immune animals within a period of hours. Rabbits were rendered immune and hypersensitive to the respective bacteria by appropriate vaccination. Half of each series were then desensitized by intravenous injections of a heavy suspension of the killed bacteria. The desensitized animals, the undesensitized ones and normal controls were then infected intracutaneously with several million lethal doses of the living bacteria. The results were clear cut. The local inflammation produced by the living bacteria in the desensitized animals was at all periods far *less* in amount, both macroscopically and microscopically, than that in the undesensitized animals *or in the normal controls*, and the tissue damage was also far less; and yet immunity to millions of lethal doses of the highly virulent bacteria remained intact. Blood cultures and bacterial stains of the local lesions showed that the bacteria were as effectively prevented from spreading from the site of infection and were as effectively destroyed in the desensitized animals as in the hypersensitive ones. It was impressively clear that even in the highly hypersensitive body the accentuated inflammatory reaction of hypersensitivity is not necessary for protection against the infection.

These studies on desensitization in acute infections were extended to tuberculous infection by Rothschild and his co-workers (26) in this laboratory. In a carefully controlled study they showed that immunized, hypersensitive animals can, by appropriate treatment with tuberculin, be desensitized so that they no longer react hypersensitively with accelerated and exaggerated inflammation and necrosis to the local injection of large amounts of virulent tubercle bacilli or tuberculin, and they tolerate with no evident symptoms the subcutaneous or intraperitoneal injection of amounts of tuberculin that would invariably be fatal for hypersensitive animals. Animals thus desensitized, together with normal controls and animals that were immunized but left hypersensitive, were infected intracutaneously, subcutaneously and in the anterior chamber of the eye (a site highly favorable for the detection of small differences in inflammatory response) with a standard dose of virulent tubercle bacilli, and the desensitized animals continued to receive their large daily dose of tuberculin which maintained the state of

desensitization throughout the experiment. Just as in the case of the acute infections described above, it was found that the desensitized animals remained as highly resistant to the proliferation and invasion of the bacilli as were the immunized, hypersensitive ones; and the former were spared from the destructive local hypersensitive effects suffered by the hypersensitive animals at the sites of infection. At the time when the organs of the non-immune control animals were riddled with tuberculous lesions, the desensitized and the hypersensitive animals showed only the minimal lesions characteristic of immunized animals, and those lesions tended, if anything, to be less numerous and less necrotic in the desensitized than in the hypersensitive animals.

Following these experiments there appeared a succession of confirmatory studies (Siegl (88); Higginbotham (22); Birkhaug (82); Cummings and Delahant (79); Boquet (89); Selter and Weiland (91); Thayer (93); Balteanu, Toma and Garaguli (94); Saenz (136); Wilson, Schwabacher and Maier (137); Branch and Kropp (92); Corper (144); Derick, Branch and Crane (90); Follis (27)). In some of these studies hypersensitivity was profoundly depressed, but not completely abolished (79) (82) (89) (93) (136) (137). In other studies (26) (22) (88) (27) (92) desensitization was complete (absence of local or constitutional hypersensitive reaction to very large amounts of bacilli or tuberculin). In all of the studies the state of desensitization was maintained throughout the entire duration of the experiment. All of these studies confirmed the fact that the hypersensitivity of immunized animals can be markedly depressed or completely abolished with no loss of acquired resistance to virulent tuberculous infection. Indeed, in most of the reports the investigators state that the resistance of the desensitized animals to the test infection was greater than that of hypersensitive controls (22) (26) (82) (89) (93) (94) (137) (92).

Bindslev (113) has reported experiments in which animals immunized with an avirulent tubercle bacillus were desensitized by injections of tuberculin and then reinfected with virulent bacilli, the desensitizing injections being continued throughout the experiment. The desensitized animals are reported to have developed in most cases more tuberculosis than hypersensitive ones. In some cases, however, they showed less tuberculosis. Only 5 desensitized and 5 hypersensitive animals that were similarly treated and studied were allowed to survive as long as a month, and 4 of these animals were complicated by pregnancy. There are various other complications in the experiments which cannot be discussed here. It will suffice to state that the animals were infected

not by the injection of a measured amount of tubercle bacilli, but by exposing them individually for various periods of time to a spray of bacilli. It is obviously impossible in such a manner to ensure equality of the inhaled infecting dose. Indeed, in the only experiment in which the lungs of some of the animals were searched for tubercle bacilli during the first 24 hours after the inhalation, bacilli were found in the lungs of the desensitized animals but not in the hypersensitive ones. Even the most ardent advocate of hypersensitivity as a mechanism of immunity would hardly claim that tissues infected with tubercle bacilli can be sterilized within a few hours; and, as a matter of fact, other hypersensitive animals of the same experiment that were allowed to live developed tuberculosis.

Recently, Willis and his co-workers (95) (96) confirmed the demonstration that during a period of two months following a test infection immunized animals that are kept desensitized show as high a degree of resistance to tuberculosis as do immunized hypersensitive animals; but they stated that if the desensitization were continued for three to six months the animals showed more extensive lesions than hypersensitive ones, and this they suggested might be due to the lack of hypersensitivity. They noted, however, that the daily injections of the large doses of tuberculin caused the animals to develop large cutaneous ulcers and to lose their appetite and become markedly emaciated (96), and they suggested that "the inanition, emaciation and ulcers of the skin all might be factors in bringing about the difference" between the amount of tuberculosis in the desensitized and the untreated animals. That resistance to tuberculosis may decline after several months under such conditions is hardly surprising without drawing hypersensitivity into the question. Indeed, to anyone who has kept guinea pigs in a desensitized state by daily injections of large doses of tuberculin it is a matter of wonder that the treated animals retain their immunity during even two months of that drastic treatment. Regardless of what may have been the cause of the final depression of immunity after some months of such debilitating treatment, all investigators have agreed that, following a standardized infection, at the time when the non-immunized controls show widespread, extensive lesions throughout the body the immunized, desensitized animals have as few and as slight lesions as have immunized, hypersensitive ones. It is therefore hardly possible to accept the view that the inhibition of proliferation and spread of tubercle bacilli in the immunized body is due to the occurrence of a rapid hypersensitive inflammation at the site where the bacilli lodge.

Willis and his co-workers (95) also reported that if normal animals were infected with tubercle bacilli and were prevented from becoming hypersensitive by the injection of large amounts of tuberculin daily from the beginning of the infection, the animals so treated showed more extensive tuberculosis at autopsy than did animals that were allowed to become hypersensitive following infection. In a subsequent study of the same type (133) in which varying amounts of tuberculin were injected, there were 21 desensitized animals which did not react definitely to 1 and 2 mgm. of tuberculin (the only test dosages to which all groups were subjected) at any test during the course of the infection. Of these, 13 had more tubercle bacilli in their lungs at death than had hypersensitive controls; but 8 of them had no more bacilli (3 had even fewer) in their lungs than had animals that were hypersensitive throughout the entire period of the experiment. Willis and Woodruff concluded that these results "point to the existence of at least a partial reciprocal relationship between the allergic state of infected guinea pigs and the number of tubercle bacilli demonstrable in their lungs." In these studies animals that had received daily subcutaneous injections of 2 cc. of undiluted tuberculin showed more tuberculosis at autopsy than those receiving only fractions of a cubic centimeter of diluted tuberculin. In none of the studies of Willis and Woodruff, however, were there any control animals that were subjected to the trauma and debilitating influences incident to the daily subcutaneous injections of 2 cc. of undiluted, glycerin-containing tuberculin. Follis (27) has repeated their experiments, adding animals that were subjected to daily injections of control solutions in comparable amounts. He, too, found that some of the tuberculin-treated animals in which hypersensitivity was prevented from appearing developed more severe tuberculosis than did the untreated hypersensitive groups; but some of those injected with the control solutions developed altogether as severe lesions, even though they possessed hypersensitivity. Since two-thirds of the animals without hypersensitivity showed no more tuberculosis than did the untreated, hypersensitive ones, and since none of the desensitized animals showed more extensive lesions than were found among the hypersensitive ones that were treated with control solutions, it seems evident that the sporadic differences in the degree of tuberculosis observed in animals injected daily from the beginning of infection are due to some factor other than the presence or absence of hypersensitivity. It was noted by Follis (27) that, as in the case of injections of tuberculin, daily injections of the control solutions likewise caused loss of appetite and emaciation. Rothschild and his co-workers (26), also, have reported

the debilitating effect of daily injections of glycerine-containing solutions which did not depress hypersensitivity.

Birkhaug (141), in a very recent study of the same type as that of Willis and Woodruff referred to above (133), found that animals that were prevented from becoming hypersensitive (skin tests always with 10 mgm. of tuberculin) during the entire period of 5 months of infection with virulent bacilli, showed less tuberculosis than controls that were allowed to become hypersensitive.

Cummings and Delahant (79), Boquet (89) and Higginbotham (22) have also interfered with the appearance of hypersensitivity by administering tuberculin from the beginning of the infection in previously normal animals. In the first two studies hypersensitivity was greatly depressed, but not abolished; in the latter, it was completely prevented from appearing. The desensitized animals in these studies showed no less resistance to the infection than did controls that became hypersensitive following infection.

Balteanu, Toma and Garaguli (138), in order to prevent the appearance of hypersensitivity, gave daily desensitizing injections of tuberculin from the beginning of immunization with attenuated bacilli, and continued the injections throughout the course (5 months) of the test infection with virulent bacilli. The desensitized animals developed less tuberculosis than did immunized hypersensitive controls. Saenz (136) and Corper (144) carried out experiments of the same type, and they, too, found that the desensitizing treatment did not interfere with the development or maintenance of acquired resistance.

It is of particular interest that Willis (97), himself, shortly after his desensitizing experiments mentioned above, carried out a study of the effect of desensitization in human tuberculosis. The results of that study agreed with those of numerous other studies on the desensitization of tuberculous patients (135) (140) (152) in that the desensitization did not depress immunity, but was beneficial. Indeed, Willis wrote: "We were agreeably surprised at the improvement experienced by more than half of the fifteen persons treated" (97).

Topley and Wilson (98), in their discussion of hypersensitivity and immunity, have objected to the conclusion that the persistence of immunity after the abolition of hypersensitivity by tuberculin desensitization demonstrates the independence of immunity and hypersensitivity, and they base their objection solely upon the ground that "the possible immunizing effect of the tuberculin itself is altogether ignored." Several others have echoed this same curiously illogical objection. It

must be obvious that even if tuberculin did possess immunizing potentialities, that fact would in no way affect the demonstration, provided by the desensitizing experiments, that immunity can function effectively in the absence of hypersensitivity. But regardless of this, Topley and Wilson were apparently not aware of the fact that numerous fruitless attempts to immunize with tuberculin had already been recorded in the literature by competent investigators (114) (71) (119). Follis (106) has recently restudied the matter, and has shown that the treatment of normal animals with the same amount of tuberculin as in the above-mentioned desensitizing experiments, and over a comparable period of time, produces no immunity whatever. This conforms with the other recorded unsuccessful attempts to produce immunity by injections of tuberculin, and invalidates the objection of Topley and Wilson.

Wilson and his co-workers (137) have recently reported studies on desensitization in tuberculosis which confirm completely the results of the numerous investigators mentioned above who have shown that desensitization does not decrease immunity. Indeed, the desensitized animals in the studies of Wilson and his co-workers showed a greater resistance than hypersensitive controls. Four separate desensitizing experiments were carried out. The authors' conclusion regarding the first experiment is: "The results of this experiment strongly suggest that the effect of continuous desensitization on the animals in group B was to limit very considerably the progress and spread of the disease." Of experiment 2 they write: "On the whole the suggestion is that animals kept continuously desensitized after infection develop a less rapid and severe type of disease than normal animals." Of experiment 3 they write: "The results of this experiment are in general harmony with the conclusions drawn from the two previous experiments. They suggest that continuous desensitization after infection tends to limit the progress of experimental tuberculosis." Of experiment 4 they write: "The results of this experiment seem to show that animals kept more or less desensitized with O.T. have a significantly longer survival time and develop less severe tissue lesions than control animals." "These results," they continue, "though perhaps more definite, are in general consonance with those described by such workers as Rothschild, Friedenwald and Bernstein, Derick, Branch, and Crane, Branch and Kropp, Higginbotham, Follis, Balteanu, Toma and Garaguli, and Saenz." It will be recalled that the experiments of all of the latter workers showed that desensitization does not decrease immunity in

tuberculosis (26) (90) (92) (22) (27) (94) (136). Curiously, in spite of the above uniformly confirmatory results of their own experiments, and without adducing any contradictory experimental evidence, Wilson and his co-workers conclude by stating that they still prefer to hold to their belief that hypersensitivity is of value in increasing resistance to tuberculosis. They give as a reason for this belief their opinion that it is a "false assumption that desensitization is complete merely because the skin reaction to tuberculin is reported as negative," and that it is not proper "to conclude that the absence of a cutaneous reaction was proof of the concurrent absence of a systemic reaction." They, themselves, made no test of the systemic reaction in their experiments, and they were apparently not aware that it has been repeatedly demonstrated that proper desensitization abolishes systemic as well as cutaneous hypersensitivity. This was clearly shown in this laboratory by Rothschild and his co-workers (26), who demonstrated that desensitized animals do not react systemically to the intraperitoneal injection of amounts of tuberculin which regularly cause fatal systemic reactions in hypersensitive animals. This was confirmed by Siegl (88), who showed that desensitized animals tolerated without symptoms the intraperitoneal injection of amounts of tubercle bacilli which produced fatal systemic reactions in hypersensitive controls; and Branch and Kropp (92) showed that desensitized tuberculous animals tolerate without symptoms even the intravenous injection of the relatively enormous dose of 2 cc. of tuberculin, which invariably caused fatal systemic reactions in hypersensitive animals. In all of these studies the desensitized animals retained their immunity unimpaired. Not only do these facts invalidate the above-mentioned objection of Wilson, but it should be clearly remembered that the standard argument for the virtue of hypersensitivity has always been that it is the accelerated and accentuated hypersensitive *local* inflammation, occurring at the immediate site where bacteria lodge, that is responsible for the inhibition of their dissemination and for their more effective destruction in the immune body; and it has been amply proved that in desensitized animals hypersensitive inflammatory reactions do not occur where the specific bacteria lodge in the tissues (28) (26) (27) (22), and yet, in those and in the numerous other similar studies referred to above, the destruction of the bacteria and the inhibition of their dissemination (28) (136) (139) were as efficacious in the desensitized as in the hypersensitive body.

The dissociation of immunity from hypersensitivity by spontaneous decline of sensitivity. It is well known that hypersensitivity gradually

wanes after a tuberculous infection has become arrested (151). Does acquired resistance decline concomitantly? Numerous investigators (105) (71) have noted that the hypersensitivity of vaccinated animals gradually wanes with the passing of time, but that their acquired resistance to virulent infection remains intact. Willis (24) carefully re-investigated this question by immunizing animals with attenuated tubercle bacilli and testing their resistance by infecting them with virulent bacilli after the lapse of one to two years, at which time their hypersensitivity had declined to so low a level that they no longer reacted to the test doses of tuberculin. He found that in spite of the "greatly reduced and practically absent" hypersensitivity, acquired resistance to virulent infection remained intact. Willis, attempting to square this result with the view that acquired resistance is dependent upon hypersensitivity, suggested that the persistence of acquired resistance after "the virtual disappearance of the hypersensitive state" was due to the observed fact that following the virulent reinfection hypersensitivity reappeared in a decidedly shorter time than was required for non-immunized, control animals to become hypersensitive (*v.* the "amamnestic reaction" discussed in a previous section of this review). The present writer has elsewhere (72) set forth objections to that interpretation, and has pointed out that the experiments provide further evidence that acquired resistance is not dependent upon hypersensitivity. This viewpoint has recently been supported by interesting studies of Schwabacher and Wilson (145) and Wells and Brooke (146). These investigators, using much smaller numbers of bacilli in the test reinfection in experiments of the same type as those of Willis, found that the return of hypersensitivity was not accelerated, as it had been in Willis' animals, but, on the contrary, it was markedly delayed and suppressed in comparison with the rate of development in similarly infected but non-immunized controls. The acquired resistance of the immunized animals, nevertheless, remained intact in spite of the delayed development of hypersensitivity. These results render invalid the suggestion of Willis that the resistance of his animals to reinfection was due to an accelerated return of hypersensitivity. When very small numbers of bacilli are used in the reinfection, as in the experiments of Schwabacher and Wilson and of Wells and Brooke, the acquired resistance conferred by the previous immunizing infection inhibits the multiplication of the bacteria to such a degree that the production of a sufficient number of bacilli to effect sensitization can be markedly delayed, for the rapidity of sensitization depends largely

upon the number of infecting bacilli (147). These studies provide striking evidence that acquired resistance not only is independent of hypersensitivity, but that its operation can actually impede markedly the development of hypersensitivity.

Hypersensitivity without immunity. Since it is well established that acquired immunity can operate effectively in the absence of hypersensitivity, and since, as was pointed out above, a heterologous hypersensitive inflammation developing where bacteria lodge has no protective effect in the non-immune body (58) (59) (60) (61), attempts have been made to determine whether specific hypersensitivity to a given bacterium can exist in the absence of immunity to that bacterium. Seibert (107), Koch and Brudnicki (117), Smithburn, Sabin and Geiger (108) and Sabin and Joyner (5) showed that animals highly sensitized by injections of tuberculoprotein, whether alone (107) (108) or together with lipides (117) (5), had no immunity to infection with tubercle bacilli. From this result it was either implied or stated that true bacterial hypersensitivity had been established without concomitant immunity. However, the animals so sensitized differed from animals with true bacterial hypersensitivity in that they did not react hypersensitively to the local injection of tubercle bacilli or Old Tuberculin (5) (110); no mention is made as to whether the local reactions to the protein were immediate or delayed; no test of passive transfer of sensitivity was made in the latter three studies, and in Seibert's (112), the sensitivity was shown to be passively transferable. Boquet, Sandor and Schaefer (41) in similar studies showed that the test reactions in animals sensitized by injections of tuberculoprotein were of the Arthus (anaphylactic) rather than the bacterial type. In view of these facts, the sensitivity produced in the above studies must be regarded as having conformed to the Arthus rather than to the bacterial type; and since the animals did not react hypersensitively to tubercle bacilli, the experiments throw no light on the question whether specific hypersensitivity to a bacterium can exist in the absence of immunity.

Mackenzie and Woo (111) and Julianelle (118) have reported that animals sensitized to pneumococcal protein by repeated injections of the protein possessed no acquired resistance to pneumococcal infection. While the description of the sensitivity to the protein in the study of Mackenzie and Woo indicated that it may have been of the bacterial type, Julianelle (42) in an extensive study of the matter was never able to produce true bacterial hypersensitivity by injecting pneumococcal protein. As in the case of tuberculoprotein, the sensitization so pro-

duced always had the characteristics of Arthus sensitivity. Unfortunately, in the study of Mackenzie and Woo there was no test of the ability of the sensitized animals to react hypersensitively to the local injection of pneumococci.

Up to the present, therefore, no one has satisfactorily demonstrated that the injection of soluble bacterial antigens will produce bacterial type hypersensitivity to the specific bacterium in the absence of immunity. Indeed, as was stated earlier in this review, there is no acceptable evidence that the antigen responsible for the establishment of bacterial hypersensitivity has yet been isolated from any microörganism.

In a filterable virus infection McKee (143) observed the appearance of hypersensitivity to living virus without immunity, shortly after vaccination with killed virus. In such animals the progress of the infection was more rapid than in normal controls.

Weissfeiler (128) has reported that animals that were sensitized by the injection of a nonpathogenic acid-fast bacillus responded with marked hypersensitive reactions to the subsequent injection of virulent tubercle bacilli, but they exhibited no acquired immunity to the infection. Boquet and Nègre (70) obtained hypersensitive reactions at the site of injection of tubercle bacilli in animals sensitized by nonpathogenic acid-fast bacilli, but the immunity of the sensitized animals was not tested. Further studies on the establishment of hypersensitivity without immunity are highly desirable.

Resumé. The characteristic form of hypersensitivity that develops as a result of infection greatly increases the susceptibility of the body to injury by the proteins of the infecting microörganisms. As a result of this increased susceptibility of the tissues to damage, inflammation is more intense at sites where the microörganisms or their proteins lodge, and severe and even fatal systemic reactions may occur when the microörganisms, or even their bland proteins, enter the blood stream of the sensitized body. Because of these effects, the hypersensitive state is a factor that has seriously to be reckoned with in the interpretation of the lesions and symptoms of infectious diseases.

Bacterial hypersensitivity differs in a variety of ways from the anaphylactic and Arthus types of sensitivity. In the latter types, both sensitizing antigen and sensitizing antibody are readily obtainable, whereas in bacterial hypersensitivity there is no acceptable evidence that the sensitizing antigen has been isolated from any bacterium, and sensitizing antibody has never been satisfactorily demonstrated in the tissues or blood of the sensitized body. Nevertheless, there are rather

persuasive reasons for believing that bacterial hypersensitivity is dependent upon antibody.

While inflammation is an exceedingly important protective mechanism, it does not follow that in the body with acquired immunity an exaggeration of the inflammatory process by means of hypersensitivity is either necessary for protection or desirable; and this particularly since the hypersensitive tendency to accentuated inflammation is inseparably bound up with an increased susceptibility of the body to injury by the bacterial products. It must also be remembered that it has been clearly shown that in the hypersensitive body not only the fixed tissue cells, but also the protective phagocytes are far more susceptible to the damaging effects of the bacterial products than are the phagocytes of the non-hypersensitive body (6) (7) (8). The study of the tissues in the above described experiments in which immunity was dissociated from hypersensitivity has shown that acquired immunity enhances so greatly the protective power of the inflammatory exudate that, instead of requiring an exaggeration of the inflammatory process, an astonishingly small amount of inflammation (far less than occurs in the non-immune body as a result of the injection of the same number of bacteria) suffices to restrain the bacteria effectively. In this, acquired immunity parallels native immunity. The body that is natively immune to a given bacterium does not respond to that bacterium with an exaggerated inflammation and destruction of tissue. On the contrary, even in the instances in which the phagocytes are essential for the destruction of the bacteria, it ordinarily requires only a minute amount of inflammation to sterilize the tissues, and the tissue damage is negligible. Studies by Rich and McKee, in which the development of inflammatory exudate was markedly suppressed by treatment with benzol, have shown in a striking manner how slight an amount of exudate suffices to sterilize the tissues in both native (116) and acquired (66) immunity.

In relation to native immunity, it is pertinent to the matter before us that there is no parallelism between the degree of native resistance and the rapidity and degree of development of hypersensitivity following infection. If hypersensitivity were a potent mechanism of protection it might reasonably be expected that the species which developed it most rapidly and in high degree would be the best protected. This, however, does not prove to be the case. For example, both the guinea pig and the white rat are susceptible to the tubercle bacillus, but the progress of the infection to a fatal termination is far more rapid in the

guinea pig, which quickly develops a high degree of hypersensitivity following infection, than it is in the rat, which does not develop tissue hypersensitivity during the entire course of the infection (120). Furthermore, Lewis and Loomis (125), in a study of the reactions of inbred strains of guinea pigs with different degrees of native resistance to tuberculosis, found that the strain which exhibited the most marked hypersensitive reactions following infection was the least resistant to the infection. Lurie (126), from a similar study of inbred families of rabbits, states that "the acquired responsiveness of the tissues to tuberculin is not definitely related to resistance." A parallel to these studies exists in the human being. The writer (148) has presented evidence, supporting the studies of other investigators, which indicates clearly that negroes have a lower degree of native resistance and are less able to develop and maintain acquired resistance to tuberculosis than whites. Their capacity for developing hypersensitivity is, however, greater than that of whites. Cummins (150) has stressed the markedly higher degree of hypersensitivity and the decidedly lower degree of resistance of the South African native as compared with European whites; and in this country Pinner and Kasper (149), from a careful study of the matter, state that their results "lead necessarily to the conclusion that infection tends to produce a high state of allergy in the American negro, but that he, unlike the white, does not readily acquire a state of increased resistance coincidently."

One of the standard arguments that immunity in tuberculosis depends upon hypersensitivity has been the fact that the antibodies present in the serum of the immunized body have not been satisfactorily shown to protect normal animals against infection. The demonstration of the effectiveness of antibodies by passive transfer in a chronic infection in which, even in the actively immunized body, the immunity is imperfect and the destruction of the microörganisms is slow and difficult, is much less readily accomplished than in the case of acute infections in which acquired immunity is more effective. For years syphilis, malaria and tuberculosis have been regarded as infections in which immunity must be "cellular," and the antibodies of no protective value. Recently, however, in both syphilis and malaria the serum of the immunized body has been demonstrated to possess the power of passively protecting normal animals (115) (122). It is reasonable to expect that in tuberculosis, likewise, proper methods may yet reveal the protective rôle of the antibodies that appear in the serum of the immunized body.

Willis and Woodruff (133), pleading the cause of hypersensitivity as a

mechanism of immunity in tuberculosis, compare this infection with smallpox, stating that in both of these infections "immunity resides in the cells," and that, in both, hypersensitivity and immunity coexist. In the case of tuberculosis the statement that immunity is "cellular" can be only a supposition, in view of the present lack of understanding of the significance of the antibodies that are present in the serum of the immunized body. In smallpox the immunity certainly does not reside exclusively in the cells, for protective antibodies, demonstrable by passive transfer, are present in the serum. But regardless of this, smallpox provides a particularly interesting example of an infection in which the available evidence speaks against, rather than in favor, of the necessity of hypersensitivity for the operation of immunity. Rivers, Haagen and Muckenfuss (134) have shown that the cells of the immunized body are resistant to vaccinia virus even *in vitro* in tissue culture, in the absence of inflammatory cells and of plasma of the immune body. There is, therefore, no reason to believe that the hypersensitive inflammation which appears at a site of infection in the intact body is in any way essential for the protection of the cells against the virus.

Many who have held the view that hypersensitivity is an essential or important mechanism of immunity have stated that one of the most persuasive reasons for that belief has been the probability that a phenomenon of bodily reactivity that is common to so many infections must serve a beneficial purpose. It may be recalled, however, that hypersensitivity to all sorts of non-bacterial proteins, resulting in conditions such as asthma, hay fever and eczema, is exceedingly common, but in no quarter are those hypersensitive reactions regarded as essential protective devices. On the contrary, every effort is made to abolish the hypersensitivity by desensitization, and the results are highly beneficial.

The studies described in this review have shown, by a variety of methods and in widely different types of infection, that neither of the two basic manifestations of acquired immunity (the inhibition of spread of bacteria and the suppression of their proliferation) are dependent upon hypersensitive inflammation. The accumulated evidence has caused a revision of the generalization that hypersensitivity is necessary for the successful operation of acquired immunity, that hypersensitive destruction of tissue is a price that must be paid for the protective effects of acquired immunity, and that tests for hypersensitivity can serve as tests for the level of acquired immunity. It is, of course, possible that

under certain as yet unknown conditions at certain periods of certain infections, hypersensitive inflammation may be necessary for protection; and in our ignorance of the true significance of all forms of hypersensitivity every effort should be directed toward the discovery of such conditions, if they exist. It is, however, of no value merely to reiterate the unproved assumption that hypersensitivity is "a stage in the development of immunity" (98). It is fair to say that, up to the present, hypersensitive inflammation has never been satisfactorily shown to be necessary for the successful operation of acquired immunity at any stage of any infection under any condition whatsoever. On the other hand, in a wide variety of infections acquired immunity has repeatedly been shown to remain intact when the capacity to react with hypersensitive inflammation has been eliminated by appropriate procedures. Since this has been demonstrated, some writers have expressed the opinion that even though the view that acquired immunity depends upon hypersensitive inflammation has not stood the test of careful examination, nevertheless hypersensitivity may embody some undefined protective mechanism which may persist when the recognized inflammatory-necrotic element has been eliminated (32). That is a viewpoint which cannot profitably be discussed until the hypothetical protective device has been defined, and has been demonstrated to be a part of hypersensitivity.

When numerous thoughtful students of a subject entertain divergent views about it, the extreme of each view is usually wrong. In the present instance one should certainly avoid both the view that hypersensitivity is always deleterious and the view that it is always essential for protection. Hypersensitivity may be regarded at present as a condition which in some instances is decidedly deleterious, in some instances exists without exerting any appreciable deleterious or beneficial effect, and in some instances may possibly serve as a useful auxiliary to the other forces of immunity. That hypersensitivity can exert deleterious effects has long been clear to everyone. That it is not essential for the successful operation of acquired immunity under a wide variety of tested conditions has now been demonstrated unequivocally. That under some, as yet undetermined, conditions acquired immunity may require the participation of hypersensitivity for its most effective operation is possible, but this possibility has not yet been established in the case of any infection. Dogmatic generalizations have long obscured unsolved problems relating to hypersensitivity. Because of its important influence upon the body during infectious diseases it is highly

desirable that efforts be continued to determine whether there exist any conditions at all under which the presence of hypersensitivity should be encouraged, and to define more precisely the conditions under which the abolition of the hypersensitive state by desensitization can be expected to be beneficial to the infected body.

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PHAGOCYTOSIS OF FOREIGN MATERIAL IN THE LUNG[1]

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The scope of the present review will be confined to a discussion of the manner in which foreign material enters the lung, its distribution within the different regions of the airways and lung parenchyma and the mechanism of disposal of such material with especial consideration of phagocytosis by the several types of cells taking part in this process. The general subject of phagocytosis, including the mechanism by which certain cells engulf foreign particles, has been so ably discussed by Mudd, McCutcheon and Lucké in a previous review (1) that an account of these more fundamental aspects of phagocytosis can well be omitted in this communication. Since the literature on the subject is concerned principally with mammals it will be assumed that the observations recorded refer to this class of vertebrates except where specifically mentioned. The various kinds of foreign particulate matter, non-living (irritating and non-irritating) and living (pathogenic and non-pathogenic) which enter the lung will be dealt with separately.

Limitations of space do not permit a detailed description of the finer anatomy of the lung. For complete accounts of this subject the reader is referred to the extensive studies of Miller (2) and more recently those of Bargmann (3), Seemann (4), Joselyn (5), Loosli (6) and Macklin (7). The nomenclature proposed by Miller for the several divisions of the bronchial tree will be employed in this discussion, namely, in the order of finer divisions, bronchiolus, respiratory bronchiole, ductus alveolaris leading into the alveolar atria into which the groups of alveoli and individual alveoli open. The ductus alveolaris and its ultimate divisions is considered as an anatomical respiratory unit or primary lobule. The continuous epithelial lining ends with the ductus alveolaris. Ciliated epithelial cells extend only to the first part of the respiratory bronchiole. The wall of the alveolus is composed of an interlacing network of capillaries supported by a framework of elastic and collagenous fibrils and contains varying numbers of mononuclear, "septal" cells. These cells include the so-called "alveolar epithelium," histiocytes such as are found in the connective tissues, as well as undifferen-

[1] Received for publication March, 1940.

tiated mesenchymal cells and fibroblasts. The capillary walls are in direct contact with the air space except for a thin ground membrane and occasional septal cells attached to the wall which do not form a continuous layer. Between the capillary loops are frequent window-like openings (pores of Kohn) which constitute direct communication between adjacent alveoli (6, 7). The plexus of lymphatics surrounding the bronchi does not extend beyond the ductus alveolaris. The only vessels in the alveolar walls are capillaries.

ENTRANCE OF FOREIGN MATERIAL INTO THE LUNG. Since the lung occupies a unique position among the internal organs of the body in that it is more constantly in contact with the external environment than any other structure within the body cavity, it became essential early in the evolution of air breathing animals that an effective means for the exclusion and elimination of noxious material from the lungs be developed. The sinuous course and the sticky mucoid surface of the upper air passages represent the initial barrier against entrance of particulate air-borne material into the lower respiratory tract. Particles penetrating this barrier come in contact with the mucus covered surface of the trachea and bronchi where they tend to adhere and, depending on the size and nature of the particle and the time element, may be largely or completely prevented from reaching the alveoli. The experiments of Barclay, Franklin and MacBeth (8) are particularly informative in this respect. These authors insufflated into the trachea of cats, bismuth carbonate dust of a particulate size 3–12 μ and determined the distribution of this substance in the lung by means of an x-ray technique which they assert is capable of detecting particles as small as 0.15 mm. They found that this material was confined entirely to the bronchial tree. None was observed to reach the alveoli although the size of the particles was such that they could easily have passed through the ducti alveolari.

That particulate matter in dry form does penetrate to the alveoli is evident from many observations both clinical and experimental. The time of exposure to air containing such material appears to be one of the principal factors in this respect (9, 10, 11). It is well known that the amount of carbon in the lungs of city dwellers increases with age. Particle size (12, 10), concentration in the air (13, 14) and depth of respiration (15) are other important determinants. Brown (16) believes that dust is retained in the lung in proportion to its wetability. The work of Jones (17) on cultures of the lungs of normal animals is especially informative. He found that bacteria of various kinds could

be isolated from the periphery of the lungs of laboratory and domestic animals whose diet consisted of hay, oats and other dusty substances. When the food was subjected to preliminary washing or changed to non-dusty material the number of bacteria found in the lungs was much diminished. In harmony with the rôle played by dust in this respect is the finding that the lungs of certain carnivors, e.g. the dog, are for the most part sterile (18) as are the lungs of human beings (19).

Many fewer data are available on the capacity of inspired fluid droplets to reach the alveoli and deal almost entirely with the inhalation of finely divided suspensions of bacterial cultures. Stillman and Branch (20) exposed rabbits and mice to such atmospheres containing streptococci, pneumococci, staphylococci and other bacteria and at short intervals thereafter were able to isolate these microörganisms from the periphery of the animal's lungs. Wherry and Butterfield (21) performed similar experiments earlier with B. enteritidis and pneumococci and were able to isolate only pneumococci from the peripheral lung tissue. In unpublished experiments Hamburger and the author (18) sprayed cultures of pneumococci into the lungs of dogs and within five minutes after completion of the procedure obtained pneumococci in pure culture from snips of tissue taken from the surface of the lung.

Whether minute droplets penetrate to the alveoli more readily than equally fine dry particles we do not know. Such data would be of great value in contributing to a better understanding of the inception of certain pulmonary infections. The nearest approach to information on this subject is provided by the work of Trillat (22) who found that exposure of small laboratory animals to very finely divided droplets of several bacterial species resulted in penetration of the bacteria to the depths of the lungs in a very short space of time. Whereas if such bacteria were mixed with talc and the animals allowed to breathe this dust, microörganisms did not reach the terminal airways. The use of a simple bacterial dust would have been much more informative since the size of the talc particles may have been such as to prevent deep penetration of the bronchial tree.

Fluid entering the lower respiratory tract is distributed in a manner quite different from that of air borne material. Such liquid material passing the barrier of the epiglottis tends to flow directly to the terminal parts of the lung, depending on the viscosity of the fluid, the position of the body and other factors less well understood (23, 24). Barclay's experiments (8) bring out strikingly the difference in distribution between air-suspended and fluid-suspended radio-opaque substances.

While the former were confined to the bronchi, the latter penetrated quickly into the alveoli. The fact that the epiglottis constitutes by no means a perfect barrier to the passage of material from the upper to the lower respiratory tracts has been appreciated only in recent years. Clinicians have long felt that the pathological condition of bronchiectasis was due principally to the implantation within the bronchi of infected exudate from the locally diseased areas in the nose and throat. But it was not until the relationship between lipoid pneumonia and oily nose drops was recognized by Laughlin (25) that this subject received special investigation. The experiments of Walsh and Cannon (26, 27) demonstrated clearly that liquids dropped into the nasal passages of animals quickly find their way into the depths of the lungs and if irritating in nature produce an inflammatory reaction in the alveoli and lung parenchyma. Webster and Clow (28) were able to produce pneumonia in mice by the nasal instillation of relatively large quantities of pneumococcus culture.

Conditions which cause failure of the epiglottis to close completely are not well understood but an important contribution to our knowledge on this subject has recently been made by Nungester and Klepser (29). These workers, by means of an ingenious technique, measured the closure of the epiglottis in rats and found that lowering the surface temperature of the animal resulted in incomplete closure. Under such conditions they found that India ink mixed with mucin and placed in the nares found its way into the lungs in considerable quantities in more than half the test (chilled) animals, while in the normal nonchilled controls very little or no ink was detected in the lungs. Using the same technique they were able to induce pneumonia in chilled rats by the intranasal instillation of pneumococci suspended in mucin in 42 per cent of the rats while infection occurred in only 13 per cent of the nonchilled controls similarly injected.

ELIMINATION OF FOREIGN MATERIAL FROM THE LUNG. The mechanism for the removal of foreign material which has not penetrated the lung beyond the terminal bronchi is under ordinary conditions very effective. This consists principally of the ciliary movement, which is capable of sweeping along particles at a surprising rate, 0.25 to 1 cm. per minute in the bronchi (30) and up to 3 cm. in the trachea (31).[2] The expulsive action resulting from the narrowing and widening of the

[2] In a series of beautifully executed experiments Proetz (32) has contributed important information on the conditions under which the functional activity of the nasal ciliated epithelium is disturbed or inhibited.

diameter of the bronchi with each respiration, the possible peristaltic movement of the bronchi (33, 34)[3] and cough all aid in the eliminatory process by way of the trachea. Extraneous material entering the alveolar units is disposed of much less easily. The principal means employed by the body for the immobilization and removal of such matter is that of phagocytosis by certain large amoeboid cells, the alveolar phagocytes or "dust cells" of Von Inns, normally present in small numbers in the alveolar spaces and mobilizable in large numbers in response to certain types of stimulation. Following engulfment of particulate material many of these cells migrate from the alveoli into the ductus alveolaris—some are then eliminated through the air passages, others pass into the lymphatic system and are deposited into the peribronchial and perivascular lymphoid nodules in the lung or carried to the hilum lymph nodes (35, 36, 37, 38). The termination of the ductus alveolaris appears to be the principal locus of entrance into the lymph channels of the bronchi and blood vessels. Certain kinds of minute particles may pass directly into the lymphatics without the intermediate step of phagocytosis. Drinker and Field (39) conclude from their experiments that this is the case with suspensions of silicate of nickel, manganese and fat particles. Hamburger and the author (18) found pneumococci in the hilum lymph nodes but not in the blood within ten minutes after spraying cultures of pneumococci into the lungs of dogs. While the usual pathway of pathogenic bacteria from the terminal air sacs to the blood is most likely by way of the lymphatics to the hilum lymph nodes, they may under special instances take a shorter route. Tuttle and Cannon (40) found that virulent hemolytic streptococci can penetrate directly from the lung of the dog into the blood stream without apparently passing through the hilum lymph nodes. There seems to be no unquestionable evidence that particulate matter, either living or nonliving, reaches the lymphatics by passage through the intact bronchial wall in regions proximal to the respiratory bronchiole (27, 41, 11).

CELLS PARTICIPATING IN PHAGOCYTOSIS. *Type of Cell.* The kind and degree of cellular reaction elicited by the presence of foreign particulate matter in the terminal airways of the lung seems to depend principally on the nature of the material. Highly irritating materials,

[3] Jarre (33) has presented good radiographic evidence for the occurrence of a peristaltic wave during expiration which moves from the more distal portions of the bronchi toward the hilum. Such peristaltic waves were not observed in severe bronchial infection. However, Gordonoff (34) was not able to confirm Jarre's findings.

living as well as non-living, produce a local exudation of blood cells, principally polymorphonuclear leucocytes together with considerable numbers of red blood cells and plasma (edema fluid). This type of cellular response characterizes the initial inflammatory reaction in the acute bacterial infections of the lung and in certain of the more chronic ones, e.g., tuberculosis. Likewise, such substances as starch and aleuronat (suspended in nutrient broth) injected into the lung call forth the same kind of cellular reaction. On the other hand, inert and relatively non-irritating materials, dusts, carbon, oil droplets, particulate dyestuffs, silica and others cause a much slower inflammatory reaction which is characterized by the mobilization of mononuclear cells, principally macrophages. Both types of cells, the polymorphonuclear leucocyte and the macrophage, are actively phagocytic but differ in their ability to engulf and digest various kinds of foreign particles. Such differences will be discussed in detail in the subsequent sections as well as the rôle played by the monocytes, bronchial epithelial cells and endothelial cells of the pulmonary capillaries.

The Macrophage. a. *Origin.* The problem of the origin of the macrophage is one which has concerned cytologists for half a century or more. It is quite outside the scope of this paper to undertake even a brief consideration of the differing ideas concerning their derivation. These concepts have been graphically stated by Gay (42). It will suffice here to present the most generally accepted views. The early work of Maximow (43), Evans (44), Aschoff (45) and others showed that these cells and their parent forms were widely distributed throughout the body both as free cells, found principally in the spleen, bone marrow, and connective tissues as well as fixed cells lining the sinusoids of the spleen, bone marrow, lymph nodes and liver. The septa of the pulmonary alveoli constitute another potential source for macrophages (46). Those in the liver appear to be a specialized type in that aside from their larger size they are by far the most actively phagocytic of the fixed macrophages.

Concerning the origin of the macrophages from the lining cells of the sinusoids of the organs above mentioned and the widely distributed reticular tissues, there is little disagreement. But opinion has varied widely as to whether these cells also represent developmental forms of certain of the blood cells, namely, lymphocytes and monocytes. While numerous investigators, Lewis and Lewis (47), Clark and Clark (48), Simpson (49), Elliott (50), Forkner (51) and others believe that the monocytes may be and often are transformed into macrophages, these

authors do not feel that there is sufficient evidence to include the lymphocytes as an earlier stage in the evolution of the macrophages. However, other workers, notably Maximow (52), Bloom (53), Taliaferro and Cannon (54), and Loosli (55) have produced what seems to the writer as convincing proof as can be obtained, that under certain conditions lymphocytes may develop into macrophages through the stages of the monocyte or polyblast. Bloom has observed such developmental sequence in carefully studied tissue cultures of mammalian lymph. These concepts are at variance with the conclusions of Sabin and co-workers (56) that macrophages arise from endothelial cells and monocytes from reticular cells but many workers feel that it is not possible to differentiate the monocytes from the various morphological and physiological manifestations of the macrophage.

b. *Alveolar phagocytes—origin.* The origin of the alveolar phagocytes, both normally present and accumulating in response to local irritation, is also a subject of controversy. Disagreement on this subject is due in part to differing concepts of the anatomy of the alveolar wall. The persistence of the older and probably erroneous view that the air sacs are lined with a continuous layer of epithelium, co-extensive with the lining cells of the bronchi, has led many investigators, who believe in the local origin of these cells, to the conclusion that the alveolar macrophages represent a transformation of these epithelial cells into free moving phagocytes. Since such a metamorphosis is unknown elsewhere in the body, it would be necessary to hypothecate, as Aschoff (57) has done, a special type of epithelial cells for this region. The structure of the alveolar wall as described at the beginning of this discussion offers no particular difficulty in ascribing a local derivation for the alveolar phagocytes. Indeed, there is a growing body of evidence to indicate that the alveolar macrophages arise locally from preëxisting septal cells. Certain authors, Briscoe (58), Sewell (59), Carlton (60), and Cappell (61), conclude that these cells develop from what they designate as the alveolar epithelium. These lining cells hypertrophy, show a cytoplasmic change from a finely granular to a foamy character and detach themselves from the alveolar wall. Other workers, Lang (46), Gardner and Smith (62), Fried (63), Lemon and Higgins (64), Tuttle and Cannon (65), and Loosli (66), ascribe to them a more general derivation, namely, from the mononuclear cells of the septum and the interstitial tissues.

Studies by the author and associates (67, 68, 69) of the cellular changes occurring during the evolution of the lesion of lobar pneumonia, both in animals and man, afforded an unusual opportunity for observing the

gradual transformation of the septal cells into free macrophages. Within 24 to 36 hours after the inception of the experimental disease in the dog, large nuclei surrounded by a darkly staining cytoplasm began to appear in increasing numbers in the substance of the septum and on its surface. These cells enlarged and those on the surface exhibited at first a fine, and later a coarsely foamy or reticulated appearance most pronounced in the unattached part of the cell. Within 36 to 48 hours these cells began to separate from the alveolar wall and entered the exudate as actively phagocytic macrophages. Whether these macrophages arise chiefly from the preëxisting lining or exposed cells or from those within the septum was not determined. Nor was it possible to estimate how many of the macrophages in these exudates developed from the local tissue cells and how many from the blood lymphocytes and monocytes mobilized in the early inflammatory exudate (55). It has been noted by practically all observers that mitotic division of the proliferating septal cells is of infrequent occurrence, although under special conditions such as the early reaction to tubercle bacilli mitotic division of the mononuclear cells of the alveolar wall has been described (70). Free binucleate macrophages, on the other hand, are not uncommon, but whether or not this represents amitotic division is uncertain.

c. *Functional activity in relation to stage of maturation.* Concerning the stage in its development at which the macrophage exhibits phagocytic activity, there exists much diversity of opinion. This applies particularly to the septal cells on the walls of the alveoli. Certain investigators, beginning with Tchistovitsch in 1889 (71) have not observed phagocytosis of foreign particles by the cells attached to the alveolar wall. Various substances introduced into the terminal air spaces have been used to determine the activity of the attached cells. Haythorn (11) employed lamp black inhaled over considerable periods of time. Ross (72) used lipiodol. Gardner (35) observed a large number of animals during inhalation of various kinds of dusts. None of these authors detected phagocytosis by the fixed cells. In our study (73) of the injection of pneumococci into the resolving lung of canine pneumonia we rarely observed microörganisms within the attached macrophages, whereas great numbers were engulfed by the free ones. On the other hand, many workers have reported phagocytic activity by the septal cells; Briscoe (58), using pigeon erythrocytes in the guinea pig, Carlton (60) with inhaled coal dust, Cappell (61) with India ink and carmine, Fried (63, 74) with pyrrhol blue and oils, Lemon and Higgins (36) with silica, Tuttle and Cannon (65) with staphylococci

and others employing various kinds of particulate matter, dyes and oils (9, 75, 76, 77). It seems not unlikely that these divergent findings may be related to the kind of material used, the form in which it reaches the lungs, possibly in some instances the character of the histological preparation and other variable factors. In this connection the experiments of Huggins and co-workers (78, 79) on the distribution of intravenously injected carbon and thorium dioxide throw considerable light on the relative phagocytic activity of macrophages in various parts of the body. They found that these materials were fixed principally in the bone marrow, spleen and liver, and relatively little in the lungs where the free macrophages are present in much smaller numbers than in the spleen and bone marrow.

Observations on the phagocytic activity of maturing macrophages in inflammatory exudates indicate that the smaller, younger cells are definitely less active than the more mature forms (58, 80, 55).

PHAGOCYTOSIS OF DIFFERENT KINDS OF MATERIAL. a. *Non-living—Naturally Inhaled.* Much attention has been given to the disposal within the lung of inert matter commonly present in the air—carbon, dusts of various kinds, and under more special conditions, stone dusts including silica and asbestos.

Carbon. Carbon appears to be one of the least irritating materials which gains entrance into the lung. The experimental studies of Haythorn (11), Mavrogordato (9), Cappell (61), Carlton (60) and others have shown that much of the inhaled carbon is deposited on the walls of the bronchi and readily eliminated. With more prolonged exposure of the animals to carbon-laden atmospheres the particles reach the alveoli in increasing numbers and are there engulfed by the alveolar phagocytes normally present. Phagocytosis under such conditions occurs slowly. Different observers have found such engulfment beginning at the end of 14 to 24 hours (60, 71). The migration of carbon laden macrophages into the lymph channels and lymphoid tissues of the lung is likewise a slow process. In rabbits killed after daily inhalation of lamp black for almost three months Haythorn (11) found none of the carbon filled macrophages in the lymphatics. Cappell (61) has found migration of such cells occurring in 15 days. These variations may well be due to the amount of carbon inhaled, since the effect of carbon on the lung tissue has been found to depend on the concentration present in the alveoli. It is only when large amounts of carbon are inhaled that irritation and consequent proliferation of the septal cells occurs. Under these conditions, extended over long periods of time, as in the experi-

ments of Schnurer and Haythorn (81) carbon filled macrophages enter the lymphatics in such numbers that obstruction to the lymph flow and subsequent fibrosis occurs. The studies of Haythorn (11) on anthracotic human lungs in Pittsburgh are particularly informative in this respect.

A quite different sequence of events is observed when carbon in aqueous solution is injected into the bronchial tree. The material, India ink (61, 64) or hydrocollargol (graphite) (82) reaches the alveoli promptly and its phagocytosis by the alveolar macrophages begins within 15 to 30 minutes. The carbon filled macrophages appear in the lymphatics within a few hours and have been observed to reach the hilum lymph nodes within 24 hours (83). Proliferation of the septal cells begins much more rapidly than is the case with inhaled carbon (61). To what extent macrophages are injured by the ingested carbon is not clear. It has been observed that macrophages containing large amounts of this material are still capable of movement and phagocytic activity (84). The polymorphonuclear leucocytes play an insignificant rôle in the phagocytosis of carbon. And since most workers make little distinction between monocytes and macrophages, data on the phagocytic activity of the former cells for carbon are not available.

MINERAL DUSTS; SILICA, ASBESTOS. On account of their importance as industrial hazards, a great deal of study has been given to the injurious effects of inhaled dusts of various kinds. The literature on this subject is so extensive that only a few of the more recent observations and summaries pertinent to this discussion can be mentioned here. Clinical studies have shown that many kinds of dusts breathed in relatively high concentration even over long periods of time are apparently harmless as far as any effect on the individual's health or detectable changes in the lung parenchyma are concerned. However, those containing silica or certain of the silicates are capable of producing marked and permanent pathological changes in the lungs. Recognition of the effects of these foreign materials and the manner in which they are dealt with by the body has come principally from numerous investigations on animals either exposed to dusty atmospheres or injected intratracheally with particulate suspensions of these same minerals. While the chemical composition of the material appears to be of chief importance in relation to harmful effect, the particulate size of the inhaled substance is a definite limiting factor in this respect. Policard (12, 85) measured the size of particles of silica and coal in the alveoli of the lungs of miners and found that 10 to 12 μ was the upper limit. This seems to be like-

wise the upper limit of the size of mineral particles phagocytable by the macrophages (85, 86, 13). It may be assumed from these observations that larger particles do not usually reach the alveolar spaces.

The characteristic lesions found in the lungs of patients dying with silicosis have been produced in experimental animals by inhalation over long periods of time and by intratracheal injection of pure mineral dusts composed of silica, serecite, borosilicate glass, quartz and others. The most complete descriptions of this pathological process have been given by Gardner and co-workers (10, 35, 87, 88) and Lemon and Higgins (86, 89, 90). Gardner (10) found that it required a matter of many months' to years' inhalation of quartz dust before any significant changes in the lungs occurred. The mineral particles are however early engulfed by the macrophages which begin to accumulate in the alveoli and then move relatively rapidly into the lymphatics and lymphoid tissues where they cause marked proliferation of the fixed tissue (mononuclear) cells. The migration of silica-bearing macrophages is much more rapid than that of macrophages containing non-siliceous dusts. These latter often remain for long periods in the alveoli. When these silica containing materials are injected intratracheally, as observed by Lemon and Higgins (89), the whole process of phagocytosis, migration into the lymphatics and proliferation of fixed tissue macrophages was greatly accelerated, presumably because a larger amount of irritating material was deposited rapidly in the alveoli. Gardner noted relatively little reaction in the alveolar wall cells. Lemon and Higgins have described early desquamation of the alveolar epithelium and migration of phagocytic cells into the alveoli. The latter authors also observed phagocytosis of silica by the attached septal cells.

The macrophages retain the silica particles and may show little functional disability for relatively long periods of time. In some of their experiments Lemon and Higgins (86) found evidence of degeneration of macrophages containing borosilicate glass after 2 months. Other authors (91, 92) have observed no evident injury of serecite-containing macrophages after 3 to 6 months. However, most workers agree that many of the macrophages ultimately degenerate and liberate the engulfed silica which is then taken up by new cells. Before such degeneration, giant cells are frequently formed (92, 88). The cause of the formation of fibrous tissue which is a characteristic feature of the silicotic nodule is not altogether clear. It is believed by most observers that it is the result of irritation and injury due to the silica liberated into the interstitial tissues by the degenerating macrophages. In harmony

with this idea is the observation that non-siliceous dusts which cause no injury to the macrophages do not produce pulmonary fibrosis (88). Others believe that the obstructed lymph flow occasioned by the marked accumulation of silica laden macrophages in the lymph channels is sufficient cause for the proliferation of fibroblasts. Such fibrosis has been observed in pronounced anthracosis (11). Lungs injured by silica are abnormally susceptible to infection with the tubercle bacillus and Gardner (35) found a high incidence of epizoötic pneumonia among his experimental guinea pigs exposed for long periods to quartz dust. The polymorphonuclear leucocytes appear to take no part in the disposal of silica.

Asbestosis is another condition closely allied to silicosis but differs from the latter in that the particles of asbestos which contain one or more of the silicates are of such a size as to preclude their penetration beyond the respiratory bronchioles.[4] This results in a somewhat different type of cellular reaction. Macrophages accumulate around the asbestos fibres, which are too large to phagocyte, and form giant cells in which the so-called asbestos bodies appear. The nature of the asbestos body, a long club shaped rod consisting of an outer layer of protein material and an inner one of changed asbestos (93, 94) suggests that it is the resultant interaction of phagocytic cells and asbestos. Fibrosis of the surrounding interstitial tissues occurs much more slowly in this condition than in experimental silicosis. The non-siliceous earths are much less irritating and while they may cause marked accumulation and retention of macrophages in the alveoli, they do not cause fibrosis. Two papers by Gardner (95, 88) contain an excellent review of the etiology and pathology of pneumoconiosis as does also the volume on silicosis and asbestosis edited by Lanza (13).

Lipoids. One of the most striking and perhaps the most spectacular example of phagocytosis in the lung is that afforded by the reaction of the macrophages to the presence of oils and fats. Study of this subject has grown out of the relatively recently recognized condition, lipoid or lipid pneumonia. Since Laughlin's original description (25) there have been a number of excellent contributions both clinical and experimental by Pinkerton (96, 77), Fried (97), Cannon and Walsh (98), Patterson (99), and Graef (100), just to mention a few. This type of pneumonia has been caused by a variety of oils and fatty materials both mineral and animal in nature and include such substances as albolene, liquid

[4] Gardner (95) states that asbestos fibres 150–200μ long have been found in the alveoli.

paraffin, cod liver oil, egg yolk, milk and cream. The vegetable oils appear to cause much less irritation than do the lipoids of mineral and animal origin. The chief sources of these foreign materials in the lungs are oily nose drops, especially in infants, and various substances taken by mouth which, in very ill and debilitated individuals or those suffering from paralysis of the upper respiratory tract muscles, find their way into the trachea past an incompletely closed epiglottis. Regurgitation and vomiting also play an important rôle in this process.

Oils entering the lower respiratory tract flow readily into the alveoli and are there engulfed by the normally present macrophages and those which gradually accumulate at the site of the foreign matter. How rapidly phagocytosis takes place is not clearly evident from studies of different investigators. Most reports indicate that this is a rather slowly progressing process—a matter of days to weeks before much of the oil or fat is engulfed. Fried (97) observed well marked phagocytosis by the end of half an hour after the experimental injection of iodized oil. The rapidity and degree to which the oil is phagocyted may well depend on its nature as shown by Patterson (99) who found that after 7 days very little of the injected liquid paraffin had been phagocyted but that after 27 days most of the oil was within the macrophages. The same was true of cod liver oil whereas transformer oil produced very little reaction in the lung and it was only after 70 days that any amount of this substance was present in the macrophages. Patterson also points out that the state of emulsification is an important factor in the phagocytosis of oils and fats.

The more innocuous oils—especially the vegetable ones—are, according to Patterson, largely eliminated without phagocytosis. But the macrophages will engulf even as non-irritating a lipoid as poppy seed oil (Lipiodol) and olive oil (99, 101, 102). Whether poppy seed oil employed in the diagnostic Lipiodol injections for outlining the bronchial tree ever causes lipoid pneumonia is doubted by most clinicians. But the very occasional occurrence of acute localized pneumonitis following this procedure raises the question of its complete innocuousness (103).[5]

The presence in the alveoli of the more irritating oils calls forth the gradual proliferation of the septal and interstitial cells but no mobilization of the white blood cells. Such polymorphonuclear leucocytes as

[5] Bloch (104) has shown that with properly adjusted temperature, injected lipiodol does not flow beyond the terminal bronchi. With this precaution he feels that any possible injurious effect of lipiodol in the lung can be eliminated.

may be found occasionally in the lesions appear to have no capacity for engulfing oil. One striking characteristic of the lipoid inflammatory reaction is the persistence of oil filled macrophages in the alveolar spaces for long periods of time. Whether or not and to what extent the macrophages migrate into the lymphatics and lymphoid tissue appears to depend on the nature of the oil. With the animal oils, cod liver oil, egg yolk, etc., and certain of the mineral oils this process may occur to a marked degree with subsequent filling of the lymph channels and the formation of fibrous tissue. Cod liver oil is particularly irritating and sometimes leads to actual necrosis of lung tissue.

The macrophages appear to have little or no capacity for digesting these foreign lipoids although Pinkerton (77) believes he has observed some digestion of animal oils and fats. Nor is there much evidence that the macrophages are appreciably injured by the ingested lipoids despite the fact that the oil may occupy a large part of the cell. Globules ranging from 2 to 30 μ have been observed (96) crowding the nucleus to one edge of the cell. These large droplets are presumably formed by the coalescence of smaller ones. However, Ross (102) noted degeneration of some of the macrophages after their ingestion of lipiodol, and the formation of giant cells in certain cases (99, 105) suggests the possibility of at least irritation of these cells. Further investigation in this connection would be valuable.

LIVING MATERIAL—BACTERIA. The varieties of bacteria which gain entrance to the lung are limited principally to those ordinarily present in the air—non-pathogenic, those producing disease of the respiratory tract, and certain microörganisms which inhabit the normal upper respiratory passages. In addition, air-borne or fluid-suspended microorganisms may be inhaled or aspirated under special and unusual conditions. While the great majority of inhaled non-pathogenic bacteria probably do not reach the depths of the lung, those which penetrate to the terminal airways appear to be so quickly phagocyted and digested by the normally present alveolar phagocytes that unless the number of microörganisms taken in is very large (17) the peripheral parts of the lung remain sterile. Very small numbers of pathogenic bacteria (subinfective doses) may well be disposed of in the same manner. Recent experiments of Hamburger and the author (18) have provided evidence in support of this assumption. Examination of the peripheral portions of the lungs of dogs into which highly virulent pneumococci were forcefully sprayed showed that while these microörganisms could be recovered shortly after the spraying, some hours later the lung tissue was sterile

on culture and showed on microscopic examination no evidence of inflammation. It was also found that transportation of the pneumococci by way of the lymphatics to the hilum lymph nodes played a rôle in this process of removal from the lung. Stillman (106) observed that while pneumococci inhaled by mice into the depths of the lung disappeared within a few hours from the lung tissue, hemolytic streptococci, staphylococci and B. Pfeiffer remained there for relatively long periods of time.

PHAGOCYTIC ACTIVITY OF THE NEUTROPHILIC LEUCOCYTES. When, however, pathogenic and, under unusual conditions, non-pathogenic bacteria are implanted in the air sacs in considerable numbers, an accessory mechanism is required to deal with the foreign irritating material. This consists of an outpouring of fluid and cells from the blood into the alveolar spaces. The initial cellular response to all the common types of bacterial pulmonary invasion, pneumococcus, streptococcus, staphylococcus,[6] B. Pfeiffer, B. Friedlander, and most of the uncommon ones, meningococcus, gonococcus, B. typhosus, etc., is predominantly neutrophilic in character. The polymorphonuclear leucocytes are actively phagocytic and engulf the microörganisms in varying numbers depending on the opsonic properties of the blood plasma. In fact, the phagocytic activity of these cells for pathogenic bacteria has been shown to be conditioned entirely by the concentration of opsonins for the specific invader. The leucocytes of animals, whose blood serum possesses no detectable opsonic action for a given bacterium, show little or no ability to phagocyte this microörganism no matter how much of the animal's serum is present, whereas these same leucocytes, when mixed with the same bacterium which has been previously exposed to the serum of an animal possessing marked opsonic properties, exhibit active phagocytosis (107).

The ability of the polymorphonuclear leucocytes to engulf pathogenic bacteria constitutes the principal factor in the so-called localizing power of the lung, i.e., a retardation of the growth of the microörganisms and of spread to adjacent tissues including the blood stream.[7] If this pro-

[6] The finding of Tuttle and Cannon (65) that staphylococci injected into the lung of a dog were phagocyted practically entirely by the septal cells may represent a special instance of the body's reaction to a microörganism of relatively low virulence for the animal species.

[7] Another localizing factor has been described by Cannon and Pacheco (108) and Rich (109), namely, agglutination and adhesion of the microörganisms to the tissue cells.

tective mechanism is defective or lacking, the animal may succumb to the infection before the body can bring other defense reactions into play. The degree to which the lung can localize implanted bacteria depends on a number of variables; the size of the infecting dose of microorganisms, the rate of mobilization of leucocytes at the site of infection, the concentration of opsonins in the blood plasma, the virulence of the microörganisms, the body temperature and other factors less well understood or not known. If the localizing power is high the infection may be terminated very quickly. If present to a lesser degree the inflammatory lesion extends but at a relatively slow rate.

The neutrophilic leucocytes possess proteolytic enzymes which are capable of digesting most kinds of engulfed bacteria. Some microorganisms, such as the gonococcus, appear to remain in the cells with little evidence of digestion. But whether such bacteria are capable of multiplication is not known. That certain kinds of bacteria are rapidly injured after ingestion even though they may appear morphologically intact has been shown by Gregg (110) with the pneumococcus. The digestive activity of the leucocytes appears to diminish much with the age of the cell (80) and the numbers of phagocyted microörganisms. We have observed that neutrophiles which have engulfed very large numbers of pneumococci are unable to digest them. Furthermore, the leucocytes may be so injured by the toxic products of the microorganisms in the surrounding medium that they lose their functional activity for either phagocytosis or digestion or both and engulfed living bacteria may be liberated (111).

The rôle played by the neutrophilic leucocytes in the body's reaction to pneumococcus infection has been studied in detail by the author and associates (112) in experimental canine pneumonia, and by Gunn and Nungester (113) in the rat. These cells accumulate early at the site of the implanted microörganisms and within a few hours begin to phagocyte the pneumococci. As the intensity of the cellular mobilization increases, more and more of the bacteria are engulfed and in the central parts of the lesion they are seen to be in the process of digestion within the leucocytes. Meanwhile many pneumococci are present in the spreading margins of the inflammatory process where they lie free in the edema fluid, such exudation being a characteristic response to the growth of pneumococci in the body. Extension of the lesion occurs as this infected edema reaches adjacent tissue by way of the smaller air passages and the interalveolar pores of Kohn. Were it not for the retarding effect of phagocytosis the lesion would spread with great

rapidity throughout the lung as indeed is observed to be the case when large doses are employed. In such lesions little phagocytosis is observed.[8]

RÔLE OF THE MACROPHAGE IN PNEUMOCOCCUS PNEUMONIA. However, despite the phagocytic activity of the leucocytes, the lesion in lobar pneumonia usually extends to involve a whole lobe and may spread to other lobes of the lung even in the animal that is destined to recover. It is evident that in order to bring about recovery from the infection the body must either increase the so-called natural means of antibacterial defense described above or bring into play a new mechanism. There is no evidence for a change in the former defense process but quite clear evidence that a new type of reaction occurs at the onset of recovery by which the pneumococci are rapidly destroyed and the disease terminated. This consists in the occurrence in the body fluids of much more potent opsonic properties and the appearance in the pneumonic exudate of increasing numbers of macrophages which arise both from the fixed tissue cells of the lung parenchyma (67) and the mononuclear cells of the blood (55).[9] In such lesions the macrophages were seen to be actively engulfing and destroying the pneumococci whereas the polymorphonuclear leucocytes at this stage of the disease, while usually still exhibiting phagocytic activity, appeared to have lost their ability to digest the intracellular pneumococci.

The same sequence of changes was observed to occur in both human and canine pneumococcic pneumonia (68) except that the evolution of the inflammatory process is much slower in the human being.

RELATIVE PHAGOCYTIC AND DIGESTIVE ACTIVITIES OF MACROPHAGES AND NEUTROPHILIC LEUCOCYTES FOR PNEUMOCOCCI. The foregoing findings gave rise to numerous questions concerning the relative phagocytic and digestive activity of the macrophages and neutrophilic leucocytes for pneumococci. The author and Van Sant (80) undertook a study of this subject employing macrophages and polymorphonuclear leucocytes secured from gum arabic and aleuronat pleural exudates in the dog. Suspensions of macrophages, which had been largely freed

[8] Rich and McKee (114) have recently brought forward striking evidence of the important rôle of the neutrophilic leucocyte in the early localization and destruction of pneumococci. These workers found that avirulent pneumococci injected into rabbits previously made leucopenic with benzol produced in general the same kind of infection as did virulent pneumococci in normal animals.

[9] C. G. Loosli in a study of the origin of the macrophages occurring in the pneumonic exudate of experimental pneumococcus pneumonia.

from neutrophiles, were employed. Tables 1 and 2 show that the macrophages not only engulf pneumococci more actively than do the

TABLE 1

Comparison of the in vitro antipneumococcal activities of macrophages and polymorphonuclear leucocytes using a concentrated macrophage suspension

A. Phagocytosis

Opsonic fluid 0.3 cc. + cell suspension 0.1 cc. + pneumococcus suspension 0.03 cc.

OPSONIC FLUID	EXAMINATION AT INTERVALS OF	DEGREE OF PHAGOCYTOSIS BY	
		Macrophages	Polymorphonuclears
	hours		
Normal dog serum	1	+++	±
	2	+++	±
	3	++	±
	5	++*	0†
1:5,000 antibody solution	1	+++	++
	2	+++	+++
	3	++++	+++
	5	no pn.	+++
1:1,000 antibody solution	1	++++	+++
	2	++	++++
	3	++++	+++
	5	no pn.	+++
Pleural fluid of immune dog	1	+++	+++
	2	+++	+++
	3	+++	+++
	5	no pn.	+++

Degrees of phagocytosis: ±, an occasional intracellular pneumococcus; +, appreciable phagocytosis but majority of pn. extracellular; ++, about as many pn. in cells as free; +++, majority of pn. in cells; ++++, all or practically all pn. in cells.

* Pneumococci beginning to grow.

† Pneumococci growing abundantly.

Note: Macrophage suspension = 80 per cent macrophages and 20 per cent polymorphonuclears. Polymorphonuclear suspension = 96 to 98 per cent polymorphonuclear leucocytes.

polymorphonuclears but show a considerably greater ability to digest these microörganisms. Such differences were most pronounced in the presence of opsonic fluid of relatively low concentration.

DEPENDENCE OF MACROPHAGES ON OPSONINS. The question as to whether macrophages in either normal or immune animals are as dependent on the presence of opsonic fluids for their phagocytic activity toward pathogenic microörganisms as are the neutrophilic leucocytes

TABLE 2

Comparison of the in vitro antipneumococcal activities of macrophages and polymorphonuclear leucocytes using a concentrated macrophage suspension

B. Intracellular digestion

Opsonic fluid 0.3 cc. + cell suspension 0.1 cc. + pneumococcus suspension 0.03 cc.

OPSONIC FLUID	EXAMINATION AT INTERVALS OF	DEGREE OF INTRACELLULAR DIGESTION BY	
		Macrophages	Polymorphonuclears
	hours		
Normal dog serum	1	0	0
	2	+	0
	3	++	0
	5	+++	0
1:5,000 antibody solution	1	+	+
	2	++	++
	3	+++	++
	5	C	+++
1:1,000 antibody solution	1	++	++
	2	++	++
	3	++++	++
	5	C	+++
Pleural fluid of immune dog	1	++	+
	2	++	++
	3	+++	+
	5	C	+++

Degrees of digestion: +, definite beginning; ++, about as many being digested as whole; +++, more partly digested than whole; ++++, only fragments of pneumococci; C, none seen (complete).

is one which has received a good deal of study. Briscoe (58), Bloom (115), Lowenthal and Misch (116), Lucké et al. (117) investigated the phagocytic activity of macrophages for both normal and specifically immunized animals for a number of kinds of bacteria and alien erythrocytes and found that in the absence of opsonins the macrophages ex-

hibited little phagocytic activity. We (80) tested macrophages from highly pneumococcus-immune dogs and found that they had no ability to engulf virulent pneumococci without the presence of opsonic fluids.

On the other hand, certain authors have brought forward evidence to indicate that during the course of infection or as a result of immunization, the macrophages may be so sensitized as to react more energetically toward the specific microörganism than do the cells of the normal animal. Lurie (118) found that bovine tubercle bacilli were phagocyted and destroyed much more quickly by the macrophages in the tissues of rabbits and guinea pigs previously infected with this microörganism than was the case in normal animals. Furthermore, he made comparative studies in vitro of macrophages from normal and tubercle-immune animals and found that the immune-animal macrophages were more actively phagocytic, not only for tubercle bacilli but also for other bacteria and inert particulate matter (119). This effect appeared to be independent of the opsonic properties of the serum. The fact that the immune body macrophages possessed a generally increased phagocytic activity suggests the possibility that they may represent a more mature type of cell than those first mobilized at the site of the primary infection. In an earlier investigation of the same general nature Clawson (120) was unable to find that the macrophages from rabbits infected with or immune to a tubercle bacillus of low virulence (B.C.G.) exhibited any greater phagocytic or digestive activity for this microörganism than did normal rabbit macrophages. He observed furthermore that immune rabbit macrophages were dependent on opsonic action especially for their ability to digest tubercle bacilli. Other workers, Rich and Lewis (121), and Aronson (122), have shown that macrophages from tubercle-immune animals, when freed from their native fluids, were more sensitive to tuberculins than were the cells from normal animals.

It seems quite possible that altered cellular reactivity as a result of exposure to invading microörganisms may depend on the kind of bacteria as well as on the animals species employed. Coggeshall (123) was unable to detect any evidence of increased sensitivity to the pneumococcus or its products in dogs recovered from experimental pneumonia or vaccinated with this microörganism, whereas Bull and McKee (124) found that rabbits could be highly sensitized to the pneumococci.

Reactions to the tubercle bacillus. While the cellular changes described in the inflammatory exudate of pneumococcus pneumonia in the dog seem to be in general the same as those occurring in response to infection with a number of other microörganisms, both in the lungs

and elsewhere, lesions produced by certain bacteria exhibit different and characteristic variations in their evolution. The acid fast bacteria and, in particular, tubercle bacilli, are in this group. The initial response of the lung to infection with this microörganism is a mobilization of neutrophilic leucocytes as described by Vorwald (125), Maximow (126) and others. These cells phagocyte the bacilli but are unable to digest them and either degenerate or are engulfed by mononuclear phagocytes which accumulate at the site of the slowly growing lesion. These cells, monocytes and macrophages, come from the blood and local tissues. Finally lymphocytes appear in increasing numbers around the periphery of the inflammatory process. The tubercle bacilli are engulfed by the large mononuclear phagocytes and undergo digestion within these cells (127) (70). Sabin and Doan (128) observed that while the macrophages destroyed the ingested bacilli the monocytes simply held them. Other workers do not make this distinction. As the lesion evolves the macrophages become the predominant cell and are designated as epithelioid cells by workers in this field who describe them as large mononuclear phagocytes containing segregated fragments of digested tubercle bacilli. The next stage in the lesion is the formation of giant cells presumably by fusion of epithelioid cells (129) (127) but some believe that they are formed by division of the nuclei within single epithelioid cells (130).[10] The functional state of such giant cells as regards phagocytic activity is not clear. In general the formation of the tubercle is analogous to that of other granulomas.

While the macrophage appears to be able to digest a great variety of living agents, there are certain pathogenic microörganisms which it can engulf but is incapable of destroying. Leishman Donovan bodies (131), lepra bacilli (132, 133), Tr. Cruzii (134), are all actively taken up by the macrophages where they remain in a viable state for relatively long periods and in some instances multiply within the cell.

Intravascular phagocytosis in the lung. The rôle played by the lung in the removal of different kinds of intravenously injected particulate material has been studied by a number of investigators. While there appears to be little question that considerable quantities of such varied substances as bacteria (135), manganese dioxide (136) and silica (137) may be held in the lung capillaries, the mechanism of this action is by no means clear. Hopkins and Parker (135) thought that injected hemolytic streptococci were phagocyted by the endothelial

[10] Gardner (88) believes that the giant cell in the silicotic nodule is formed by nuclear division.

cells of the pulmonary capillaries but could not be sure of this. Likewise Drinker and Shaw (136) observed particles of manganese dioxide in the capillary endothelial cells but found that the bulk of this material was held only temporarily in the lung (138). Gardner and Cummings (137) noted that many particles of injected silica were trapped in the lung capillaries and then phagocyted by large mononuclear cells but they saw particles within giant cells in the alveolar walls. Mole (139) also observed marked phagocytosis of stained (marked) red blood cells in rabbits' lungs but could not be certain of the origin of the phagocyting cells. Neither Cappell (61) nor Westhues (140) could detect any phagocytic activity of the endothelial cells of the pulmonary capillaries for India ink, or collargol. The experiments of Wislocki (141) contribute valuable information on this subject. He found that considerable quantities of finely suspended India ink of a particulate size of 1–3μ, injected intravenously were caught in the lung capillaries but not in the systemic capillary system except the liver and spleen, which suggests that retarded blood flow in the lung plays an important rôle in this process. Wislocki believes that phagocytosis plays no rôle in the trapping of carbon in the lung which material he observed to be present in the capillaries in small plugs. After a time, mononuclear phagocytes collected around the carbon and began to engulf it but the endothelial cells took no part in this process. Thus the evidence presented and that of other workers not cited, indicates that the capillary endothelium of the lung exhibits little or no phagocytic activity for circulating foreign particles.

PHAGOCYTOSIS BY THE EPITHELIAL CELLS OF THE BRONCHIAL MUCOSA. Whether the epithelial cells of the bronchial tree possess any ability to engulf foreign material is another subject of controversy. The majority of workers believe that these epithelial cells are non-phagocytic (11, 59, 38, 96, 9). Gardner and Cummings (94) found no asbestos particles in the walls of the trachea and bronchi even after two years of constant experimental exposure to this material.[11] However, certain investigators, on the basis of carefully conducted experiments, have come to the opposite conclusion. Ropes (142) found that the vitally stained cuboidal ciliated cells of the lungs of rabbits exposed to carbon dust contained many carbon particles. The long columnar cells showed little or none of this material and no phagocytosis by any of these cells

[11] Tchistovitch (71) found that neither the living cells of the frogs' lungs nor those of the swim bladder of the fish exhibited phagocytic activity for particulate matter.

was observed if the India ink was injected in fluid suspension. Duthrie (143) considered that he had demonstrated phagocytosis of carbon by the cells of the bronchial walls but his work was not nearly as convincing as that of Ropes. Smith, Willis and Lewis (144) studied tissue cultures of chick embryo seeded with tubercle bacilli and observed phagocytosis of the bacilli by the non-ciliated epithelial cells of the bronchi and the lining cells of the alveoli. Carlton (145) carried out a well controlled study of the phagocytic activity of the columnar epithelial (nonciliated) cells of the uterine horns and vagina in the rabbit. He was able to demonstrate that these cells engulfed graphite, staphylococci and carmin particles. Castaneda (146) has recently shown that after the intratracheal injection of typhus Rickettsiae the bronchial epithelium becomes parasitized with these microörganisms. The foregoing experiments indicate that under special circumstances and in special loci, epithelial cells may exhibit phagocytic activity. However, regardless as to whether or not certain cells of the bronchial epithelium are capable of phagocytosis, it seems evident that they play a very insignificant rôle in the removal of foreign material by this means.

Significance of phagocytosis in the lung. While the process of phagocytosis represents the most effective means the body possesses for disposing of foreign matter which has penetrated into the terminal areas of the lung, it fails in many instances to protect the body against the noxious action of such extraneous material. This is particularly true of those substances which are not susceptible to the digestive enzymes of the phagocyting cells. It is not inconceivable that the transfer by the macrophages of the irritating dusts (silicates) and lipoid material from the alveoli to the parenchymatous tissues may ultimately result in greater injury to the lung than if such substances were allowed to remain in the air sacs where some possibility of their expulsion exists. However, the progressive accumulation of particulate material in the alveoli, even though non-irritating, would undoubtedly lead finally to serious interference with the respiratory function of the lung. The protection afforded by phagocytosis against invading microörganisms is in general much more adequate since the phagocyting cells possess enzymes which are capable of digesting most kinds of bacteria. In some instances, when the virulence of the microörganism is relatively low or the numbers of implanted bacteria even though highly pathogenic, are very small, destruction of the bacteria may be accomplished entirely by the neutrophilic leucocytes which are the first to be mobilized at the site of infection (18). Even when the infection becomes well established

it is the polymorphonuclear leucocyte which constitutes the body's first line of defense against the pathogenic invaders. This is particularly true in the acute pulmonary infections. The neutrophiles act to check the spread of the infecting bacterium until the more slowly accumulating macrophages take over the function of phagocytosis and digestion. Since these cells possess more potent intracellular enzymes than do the neutrophiles, they will, under favorable circumstances, in conjunction with the humoral immune substances, bring about either complete destruction of the invaders, as in pneumonia, or sharp localization of the infectious process characteristic of the favorably progressing tuberculous lesion.

The important rôle played by the macrophage in the process of recovery from infectious disease has been demonstrated by a number of investigators; Gay and co-workers (147, 148, 149) for the hemolytic streptococcus, Cannon et al. (150, 65, 151) for staphylococcus and B. coli, Robertson and associates (67, 68, 69, 152) for the pneumococcus, Maximow (153), Sabin and Doan (128), Lurie (70), and others for the tubercle bacillus, Taliaferro and co-workers (54, 154, 155) for protozoan infections, to mention only the most extensively studied pathogenic microörganisms. The only generally recognized mechanism by which the macrophage causes destruction of these disease producing agents is that of phagocytosis and intracellular digestion. Gay (147) found that extracts of macrophages and supernatant fluids from macrophage exudates would cause lysis of hemolytic streptococci. But this effect has not been shown for other pyogenic cocci. It is not unlikely that the free macrophages are capable of producing other substances which affect bacteria outside the cells but little definite information is available on this subject.

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THE BLOOD SUGAR: ITS ORIGIN, REGULATION AND UTILIZATION

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1. Carbohydrate Metabolism as a Dynamic Balance. As will appear from the evidence to be reviewed, it is well established that sugar is supplied to the blood by the liver when it is not forthcoming from the gastro-intestinal tract or other exogenous sources. The blood sugar is chiefly utilized in the extra-hepatic tissues. It is also known that in every species of animal and under all experimental conditions in which it has been done the removal of the liver leads to a rapid, profound, and fatal hypoglycemia, which can be alleviated by the continuous administration of relatively large amounts of dextrose. It may therefore be stated as axiomatic that the blood sugar level represents a dynamic balance between the rate at which sugar is entering the bloodstream from the liver and from any exogenous source, and the rate at which it is being removed from the blood by the tissues of the body. Thus a rise in the blood sugar level may result either from an increased rate of sugar supply or from a decreased rate of sugar utilization, or from both together. Conversely, a fall in the blood sugar level may be due to decreased supply, increased utilization, or both. Nor is it possible to tell, from the mere change in blood sugar level, which factor is responsible unless one factor is controlled or eliminated while the other is observed. These considerations also apply to the various other criteria of carbohydrate metabolism with which we shall deal.

This simple but fundamental conception dates back to Claude Bernard. Nevertheless it seems necessary to emphasize it here from the outset, for it has all too often been ignored in the development of some of the most widely accepted views of carbohydrate metabolism. While the concept of the dynamic balance may seem sufficiently obvious when applied to the blood sugar level as above, it has often been overlooked in the consideration of such matters as the D:N ratio, the respiratory quotient, ketosis and carbohydrate balance studies. In discussing

these topics we shall, therefore, have frequent occasion to allude to this basic error, and to attempt to avoid it in our own interpretations.

PART I. ORIGIN OF THE BLOOD SUGAR. *2. The Site of Formation of the Blood Sugar.* The secretion of sugar into the blood by the liver of the fasting animal was demonstrated by the brilliant pioneer work of Claude Bernard (10). The inadequate chemical and physiological methods available to him and to his contemporaries initiated an involved controversy which ultimately partly obscured his original conceptions (289). Subsequent observations on the development of hypoglycemia following hepatectomy in birds (139) (190), and after total abdominal evisceration in mammals (172) (207), again directed attention to the liver as the source of the blood sugar. It remained for F. C. Mann (177) to devise the first practical method for complete hepatectomy in dogs. Mann's three-stage operation was simplified to a two-stage procedure by Markowitz and Soskin (183) (240), in which form it has been possible to apply it to dogs, cats, rabbits, guinea pigs and monkeys (181). More recently, Markowitz, Yater and Burrows (184) have described a feasible one-stage method of hepatectomy which, with slight modification, makes total abdominal evisceration of the dog (255) and rabbit (195) a relatively simple matter. It is upon these methods that much of the recent progress in evaluating the hepatic and peripheral aspects of the dynamic carbohydrate balance has depended.

The work of Mann and collaborators finally established the liver as the prime factor for the maintenance of the normal blood sugar level. The presence of appreciable quantities of glycogen in the muscles of their hepatectomized dogs during profound hypoglycemia led them to conclude that muscle glycogen is incapable of sufficiently rapid conversion to glucose to play a significant rôle in maintaining the blood sugar level (19). This conclusion was confirmed and extended by Soskin (237) who demonstrated that the known hyperglycemic agents, epinephrin, ether anesthesia, and asphyxia, have no influence whatever on the falling blood sugar level of liverless dogs. More recently Houssay and his associates have found a similar absence of the hyperglycemic action of extracts of the anterior pituitary gland, in hepatectomized toads (34) (119) and dogs (120). Soskin concluded that muscle glycogen is not an available source of blood sugar in the absence of the liver, and that the liver is the sole source of supply for the glucose in the blood in the fasting organism. He pointed out, however, that muscle glycogen might give rise to blood sugar in the intact animal by indirect means;

that is, when lactic acid is produced in excess by the muscles, reaches the liver by way of the blood stream, and is there converted into glycogen. This so-called lactic acid cycle was later investigated and elaborated by Geiger (84) (85) (86), Himwich (109), Cori (42) and others.

3. Use of the Diabetic Organism in the Study of Gluconeogenesis. The chief medium of research as regards the precursors of sugar in the animal organism and especially as regards the amount of sugar which may be derived from them, has been experimental diabetes. More specifically, the extra-excretion of sugar in the urine of the depancreatized or phlorhizinized animal following the administration of a known amount of a foodstuff, has been taken as the measure of the extent to which that substance may give rise to blood sugar. The rationale of this method is based upon *evidence* that the diabetic organism cannot store much carbohydrate, and upon the *assumption* that the newly-formed sugar cannot be utilized but is excreted quantitatively into the urine. The empiric usefulness of the information derived from the method has, in turn, tended to raise the latter assumption concerning the nature of diabetes to the dignity of a proven fact. The evidence to be reviewed later shows that the diabetic organism can and does utilize large amounts of blood sugar. This does not entirely invalidate the data obtained in feeding-excretion experiments. It does necessitate a brief consideration of the nature of the diabetic disturbance in order that such data may be safely interpreted.

From the standpoint of the dynamic balance and avoiding post hoc propter hoc reasoning, the hyperglycemia and glycosuria of diabetes may be due to either or both of two possible disturbances: *1*, a loss of the ability to utilize blood sugar, accompanied by a normal rate of gluconeogenesis (the Non-Utilization Theory); *2*, an increased rate of gluconeogenesis accompanied by a normal rate of utilization (the Overproduction Theory).

When these two possibilities are represented in a simple mechanical analogy, as in figure 1, it is readily seen that the addition of an extra quantity of sugar *in either B or C* will result in an extra overflow through the kidney in addition to the excess which is already being excreted. Thus, the more or less quantitative excretion of administered sugar by the severe diabetic does not constitute proof for or against either theory. Similarly the administration of a given amount of a non-carbohydrate precursor of blood sugar will result in an extra excretion of sugar on the basis of either theory. However, here the similarity ends. For, if the non-utilization theory is correct, the extra excretion of sugar must

be a quantitative measure of the extent to which the precursor is converted into sugar. While, if the overproduction theory is correct, there is the possibility of some coincident variation in the rate of utilization so that the amount of extra sugar which is excreted may represent only a portion of that which is derived from the precursor. It must also be remembered that, on the basis of either theory, the extra excretion of sugar following the administration of a precursor will depend upon the capacity of the liver for gluconeogenesis from that particular precursor over and above that which is already proceeding. This is particularly apt to apply in the case of overproduction, where gluconeogenesis from one or more of the endogenous non-carbohydrate precursors may already be near or at its maximum.

4. Gluconeogenesis from Protein. (*a*) *In the depancreatized animal.* The origin of blood sugar from ingested carbohydrates or from liver

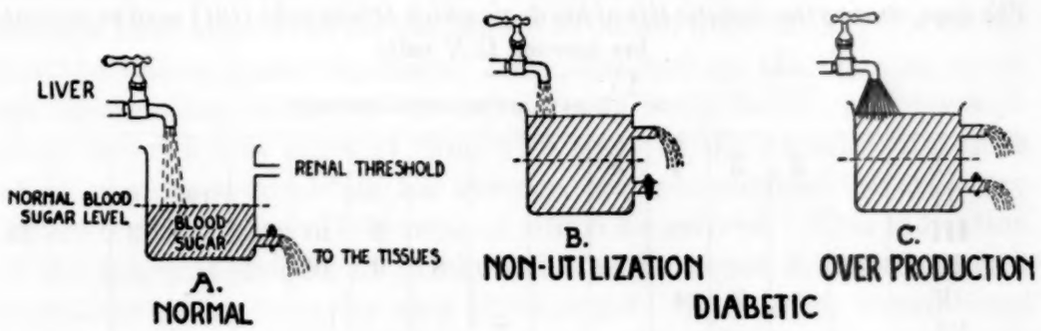


Fig. 1

glycogen resulting from carbohydrate feeding, requires only passing mention. A consideration of the non-carbohydrate precursors of blood sugar in the fed or fasting animal properly begins with the pioneer work of Mering and Minkowski (188) on depancreatized dogs. From the extensive glycosuria exhibited by these animals, and especially from the fact that administered sugar was excreted more or less quantitatively in the urine, Minkowski (191) concluded that depancreatized dogs have lost the power to utilize carbohydrate. He further observed that, for a varying number of days during an intermediate stage of his experiments, the amount of glucose excreted by the animals bore a fairly constant relationship to the amount of nitrogen excreted in the urine. This relationship, which was not affected by varying the amount of protein in the diet, was named the D:N ratio. Minkowski interpreted this ratio to mean that protein was the sole source of the excreted sugar, and that a constant proportion of the protein was being converted

into sugar. It is apparent from our previous discussion of the possible disturbances in the diabetic organism, that Minkowski's conclusions from the glycosuria and the quantitative excretion of administered sugar were not justified. If, however, the D:N ratio were really constant, it would be difficult to conceive of a rate of sugar utilization (except zero utilization) so unvarying in different animals and under different conditions as to make the ratio possible. A critical examination of the constancy of the D:N ratio is therefore in order.

On 31 experimental days, in 9 depancreatized dogs fed on meat, Minkowski obtained D:N ratios which varied from 3.16:1 to 2.62:1, with an average of 2.8:1. The experimental days which he used to establish the average ratio were admittedly selected, since a record of all the experimental days on any single animal would show D:N ratios

TABLE 1

The days, during the diabetic life of his dogs, which Minkowski (191) used to compute his average D:N ratio

DOG	DAYS AFTER PANCREATECTOMY														
I		2	3	4	5				9	10					
II												12	13		
III									9	10	11		13		15
IV								8	9	10					
V			3	4											
VI							7								
VII						6		8			11				
VIII					5	6	7	8							
IX											11	12	13	14	15

much higher than 2.8:1 to begin with, and ratios which fell progressively below this figure as the exitus of the animal was approached. The high initial D:N ratios were discarded on the basis that they represented the pouring out of preformed glycogen stores. The low D:N ratios towards the end of the experiments were disregarded because of the poor condition of the animals at that time. The reasonableness of these objections to the results of the first and last few days of each experiment cannot be denied. But a closer examination of his data makes it apparent that the experimental days were selected in a much more arbitrary manner than we have been led to believe by those who have trustingly accepted Minkowski's average as a physiological constant.

Thus, the selected data in any given experimental animal began as

early as the second day of diabetes or as late as the twelfth day, and ended as early as the fourth day of diabetes or as late as the fifteenth day. The days reported in some experiments, moreover, are not consecutive, some days being omitted for no stated reason. It must be apparent that any desired average D:N ratio might have been obtained by such arbitrary selection of experimental days, picked from experiments in which the D:N ratios fell progressively from high to low values.

This criticism is supported by other results of Minkowski, reported in the same paper but not included in the figures from which he obtained his average D:N ratio. In comparing the initial D:N ratios obtained from well-nourished and poorly-nourished animals, he recorded ratios of 2.04, 2.43, 1.62 and 2.24 on the third, fourth and fifth days of diabetes in the latter animals. It is difficult to understand why Minkowski did not attempt to correlate these results with the data from which he computed his average ratio. The poor nutrition of these animals might perhaps have accounted for the failure to obtain high initial D:N ratios. But the values uniformly below 2.8:1 obtained on the days in which the approaching demise of the animal was not a factor, and on days which coincided in point of time with some of the experimental days which were used to obtain his average, serve to confirm the arbitrary nature of the average D:N ratio at which he arrived. This indication of the inherent defects in Minkowski's work is not intended to cast aspersions on his integrity as a physiologist. It must be remembered that Minkowski, working before the days of insulin, had to deal with acutely diabetic dogs suffering from the effects of a recent anesthetic and operation.

Pflüger (209), Embden (71) and others subsequently reported that they had failed to obtain fixed D:N ratios at the Minkowski level. Their work was criticized on the assumed grounds of the poor condition of their animals, or of incomplete pancreatectomy. Such criticism, however, cannot be leveled at the work of Macleod and Markowitz (171) who used depancreatized dogs maintained in excellent condition by the use of insulin. After the withdrawal of food and insulin from such animals (which by subsequent post-mortem examination were shown to be completely depancreatized) they obtained D:N ratios far below 2.8:1, after the first few days of the experiment had elapsed. Chaikoff and co-workers (37) reported similar results and found (as noted by Minkowski) that the D:N ratio was generally higher in fat than in lean dogs, and varied decidedly in the same animal, according to its nutritional condition at the time of the experiment.

In 1930 Rapport (217) reviewed the extensive literature on the D:N ratio in addition to the above, and was not able to reconcile the large variations which had been reported. In the same year Soskin (239) published a comprehensive re-investigation of the D:N ratio in depancreatized dogs, using the advanced technique made possible by the discovery of insulin. This work was done on depancreatized dogs, completely recovered from operation by the use of insulin, and presenting well-healed and non-infected wounds. The animals were maintained on a low-calory, protein diet, and the absence of islet tissue was verified by post-mortem examination. In contrast to Minkowski's animals, they survived the withdrawal of insulin for as long as 5 weeks, during which they usually remained bright and active although losing weight. The observations on the D:N ratio comprised 138 unselected days in 10 dogs, in contrast to Minkowski's 31 selected days in 9 dogs. The distribution of the D:N values obtained was as follows:

TABLE 2

Distribution of D:N ratios during 138 unselected days, in 10 depancreatized dogs (Soskin (239))

	RANGE OF THE D:N					TOTAL DAYS
	Over 3.16	3.16-2.62 (Minkowski's range)	2.61-2.00	1.99-1.00	Less than 1.00	
Number of experimental days on which obtained	15	33	43	36	11	138

It may be seen that although some D:N ratios similar to Minkowski's were obtained, there is nothing to indicate that such values have any particular significance. In general the D:N ratio tended to be high at the beginning of each experiment and to show a progressive fall as the animals lost weight and their stores of adipose tissue were depleted. This serves to explain the different D:N ratios reported by previous workers. The fact that some animals maintained D:N values far below 2.0:1 for as long as 18 days, precludes the appellation of "premortal" which some writers have used to avoid consideration of all ratios below the Minkowski level (40).

It is clear that, if Minkowski's interpretation of his ratio is accepted, the progressively lower ratios obtained later in the experiments signify the utilization of increasing amounts of the sugar arising from protein.

If, on the other hand, the low ratios obtained later in the experiments represent the true extent of gluconeogenesis from protein, the higher Minkowski values must mean that sugar is being formed from fatty acid as well as from protein. In either case there remains no basis for concluding that sugar is derived solely from protein, or that none of the sugar so formed is utilized by the diabetic organism. It is permissible to conclude that sugar is derived partly from protein, but it is impossible to say to what extent this occurs.

(*b*) *In the phlorhizinized animal.* Similar conclusions may be drawn as regards the significance of the D:N ratio of 3.65:1 *formerly obtained* (53) by some investigators in so-called phlorhizin diabetes. There is an added difficulty in interpreting this type of work in that there is no standard for judging the experimental preparation, comparable to histological demonstration of complete pancreatectomy in operated animals. It is obviously fallacious to account for different D:N ratios obtained with phlorhizin in different animals and by different workers (217) by saying that some of the animals were not completely phlorhizinized because they did not yield D:N ratios of 3.65:1. But even if one were to accept the latter figure as correct, one would still have to explain the difference between it and the Minkowski ratio of 2.8:1. There is no factual basis for concluding that phlorhizin alters the biochemical processes in such a manner as to allow a larger proportion of the protein molecule to be converted into sugar. And, if a constant proportion of the protein molecule is convertible, then either the depancreatized animal always utilizes a significant fraction of the sugar derived from protein, or the phlorhizinized animal must be forming sugar from fat as well as from protein. We must add to this the evidence that: (a) Insulin has been obtained from the pancreas of dogs after prolonged and maximal phlorhizin treatment (204). (b) The phlorhizinized dog after nephrectomy is quite normal as regards its blood sugar level, its respiratory quotient, and the rise in the R.Q. following glucose administration (56) (72). (c) The ingestion of sugar by the intact phlorhizinized animal results in the retention of glucose, which has an antiketogenic and protein-sparing action, and causes a rise in the R.Q. comparable to that which occurs in the normal dog (56) (198) (203) (277) (278). (d) Carbohydrate balance experiments on eviscerated phlorhizinized dogs show that the extra-hepatic tissues of these animals utilize sugar at the same rates as do the tissues of normal animals at similar blood sugar levels (251). It is, therefore, quite apparent that the D:N ratio of phlorhizin-treated animals cannot sig-

nify that no sugar is being utilized, or that the sugar which is excreted represents either the partial or total amount which is being formed from protein alone.

5. Gluconeogenesis from Fat. We have seen that the D:N ratio does not warrant the conclusion that sugar cannot be utilized by the diabetic organism. It will be shown later that there is ample positive evidence that carbohydrate utilization is not much impaired. The dextrose component of the D:N ratio therefore represents the net remainder, when the extra-hepatic utilization is subtracted from the hepatic blood sugar formation. Once this is granted, it must inevitably be concluded that the breakdown of protein cannot supply sufficient sugar for both excretion and utilization, and that the liver forms sugar from fatty acid as well as from protein. The long controversy on this subject in the past was made possible only by the dearth of positive evidence of carbohydrate utilization, and the fact that the calculations of the "non-oxidation" proponents were always made on the assumption that none of the sugar formed by the liver was utilized in the diabetic organism. The evidence for gluconeogenesis from fat on this basis has been comprehensively reviewed by Geelmuyden (83) and Macleod (170) and will not be discussed here. We will consider the more recent evidence, in which the utilization of sugar is taken into account, or in which it is excluded as a factor by direct observations on the formation of sugar in the isolated liver.

From carbohydrate balance experiments on rats and rabbits, Cori (42) calculated the quantities of blood sugar which the liver would have to secrete in order to account for the epinephrin hyperglycemias which he observed. It was apparent that the liver could not supply the necessary amounts of blood sugar, unless the greater portion of that sugar were derived from fatty acids. Since he considered the latter process unlikely, he marshalled certain evidence from which he concluded that epinephrin hyperglycemia is chiefly due to a decreased utilization of sugar by the muscles. Soskin and co-workers (259) tested Cori's findings and conclusions by using measurements of bloodflow to convert arteriovenous blood sugar differences into amounts of sugar retained by the muscles per unit of time. They could find no indication that epinephrin decreased the utilization of sugar. Similar negative results were obtained when the experiments were repeated with the coöperation of Essex, Herrick and Mann (245). Still more recently, Himsworth and Scott (108) have reported that epinephrin actually increases the utilization of sugar by the peripheral tissues. Although the significance of

the data which they interpreted to mean an increased sugar utilization may be open to question, their results certainly confirm the fact that epinephrin does not cause a decreased utilization of sugar. From these considerations it must be concluded that epinephrin hyperglycemia is a hepatic affair and that, if Cori's above mentioned calculations are correct, they indicate the occurrence of gluconeogenesis from fat in the liver, under the influence of epinephrin.

Recently Young (287) (288), using figures for the sugar utilization of the extra-hepatic tissues of normal and depancreatized animals, which are available from hepatectomy experiments, has calculated that sugar must be formed from fatty acids in the livers of both types of animal.

It is apparent that the indirect demonstration of gluconeogenesis from fat is not difficult, once the masking effect of the simultaneous utilization of the formed carbohydrate is taken into account. Those who have not accepted the evidence that the diabetic organism utilizes carbohydrate have, of necessity, denied the possibility of gluconeogenesis from fatty acid on the basis of indirect evidence, involving *a*, the D:N ratio; *b*, ketosis, and *c*, the respiratory quotient. It is therefore necessary to anticipate the discussion of carbohydrate utilization, with a consideration of the alternative explanations of these metabolic criteria.

a. The D:N ratio. The administration of fat to experimentally diabetic animals has usually not resulted in sufficient excretion of extra-sugar to indicate gluconeogenesis from fatty acids, when the calculations were made on the basis of the classical interpretations of the D:N ratio (217). This is not surprising when it is remembered that these interpretations, by their very nature, practically exclude the possibility that such calculations might yield positive results. Even so, extra sugar might still be excreted if the experimental animal could make additional amounts of sugar over and above that which it is already forming from endogenous protein and fat, including the amount which is being utilized during the experiment. But this involves the unwarranted assumption that the capacity of the liver for gluconeogenesis from fat has not been reached before the fat is administered. The fact that this is not the case for protein has no bearing, for it happens that fat is the only stored food substance present in practically unlimited amounts as far as the daily requirement of the body is concerned. It might therefore be expected that it would be used to capacity when the liver is forming sugar at an uncontrolled rate. From the practical

standpoint, the experimental procedure to test the extra sugar excretion involves the administration of fat to the diabetic animal on the fourth or fifth day after pancreatectomy, after the withdrawal of insulin, or after starting phlorhizination. At this time the animal is suffering from acute diabetes with ketosis, and the administered fat makes him even more sick. In certain experiments in which some extra excretion of sugar after fat administration was reported (238) the animals shortly died. In order to obtain positive results by this method it is apparently necessary to exceed physiological limitations, to a degree incompatible with life.

b. Ketosis. The ketosis in diabetes and other conditions has been explained in two chief ways. Those who accepted the Minkowski D:N ratio and the orthodox interpretation of the R.Q. have regarded the ketone bodies as abnormal toxic products of incomplete fat oxidation, which accumulate because of the lack of simultaneously oxidizing carbohydrate (111) (225) (232) (284) (285). Others have regarded the ketone bodies as essentially normal intermediate metabolites, appearing during the conversion of fatty acid to sugar, and accumulating in abnormal amounts whenever the rate of gluconeogenesis from fat becomes excessive (83) (170) (206) (239). Parenthetically, it should be mentioned that it is agreed that unimportant amounts of ketone bodies may also arise from certain amino-acids. Beginning with Embden and coworkers (69) many investigators demonstrated that the liver was the chief source of the ketone bodies, and that the fatty liver forms more ketone bodies than one rich in glycogen (151) (216). It was also found that livers from depancreatized or phlorhizinized animals produced more ketone bodies than livers from normal animals (70).

The crucial difference between the two viewpoints outlined above was that the first theory ascribed the ketosis of diabetes to an inability of the extra-hepatic tissues to dispose of the ketone bodies, while the second hypothesis postulated a rate of hepatic formation in excess of the existing normal ability for peripheral disposal. The work of Chaikoff and Soskin (38) strongly supported the latter viewpoint. They showed that the tissues of the eviscerated-depancreatized dog disposed of injected sodium acetoacetate with equal facility as compared to the extra-hepatic tissues of the normal dog, and that the apparent slower rate of disposal after injection into the intact diabetic animal was due to excessive amounts of endogenous ketones with which the tissues of the animal were already dealing. More recently, the same viewpoint has been firmly established by the outstanding work of Mirsky and

collaborators. Using a ketogenic extract of the anterior pituitary gland, they demonstrated that the ketones so formed arise only in the liver (192), that insulin which suppresses this ketosis does so by acting on the liver and not on the extra-hepatic tissues (193), and that the rate of disposal of the administered sodium salt of β-hydroxybutyric acid by the extra-hepatic tissues was independent of the amounts of sugar being utilized by those tissues (196) (197) (273). This and other evidence (62a) make it no longer possible to regard ketosis as indicating a lack of carbohydrate oxidation by the muscles. It is clear that in diabetes the ketone bodies, like the blood sugar, are utilized by the extra-hepatic tissues, but are produced by the liver at rates which exceed the power of the tissues to dispose of them.

c. The respiratory quotient. The non-protein R.Q. of about 0.7, which is usually obtained in both the fasting and the diabetic states, has been interpreted to mean that fat alone is being oxidized. Since the conversion of fat to carbohydrate occurring simultaneously with the oxidation of fat would result in lower R.Q. values, it has been concluded that this conversion does not occur. But, it is becoming increasingly more evident that the respiratory quotient of the whole body, like the D:N ratio, cannot be regarded as the index of a single process. The orthodox interpretation of the non-protein R.Q. of about 0.7, involves the tacit assumption that the only vital processes (aside from protein catabolism) which are in progress and which ultimately consume oxygen and give rise to carbon dioxide, are those associated with the oxidation of fat. Yet there is very satisfactory evidence that other processes which require oxygen or yield carbon dioxide are taking place under those conditions. It is generally agreed, for example, that the brain derives its energy solely at the expense of carbohydrate, and yields an R.Q. of about 1.0 at all times (3) (5) (57) (110) (156). This high R.Q. must be balanced by a correspondingly low one, if the composite R.Q. of 0.7 is to mean anything at all. Indeed, this principle of different R.Q.'s forming a composite total R.Q. is generally used to interpret an R.Q. over 1.0, on the basis that the oxidative R.Q. is being elevated by a simultaneous conversion of carbohydrate into fat (220). But there is no evidence that the high R.Q. due to the latter process cannot be one of the components of any R.Q. under 1.0 (185) (217).

Similarly, Cathcart and Markowitz (35) and others have shown that the oral administration of 50 grams of glucose to the fasting human causes a leisurely rise in the R.Q. to values somewhat less than 1.0, while the administration of equivalent quantities of sucrose, galactose,

levulose or dihydroxyacetone causes a prompt rise in the R.Q. to values above unity. Since the rates of absorption of the latter substances from the gastro-intestinal tract cannot account for the difference, and since it is known that they must be converted to dextrose by the liver before they can be utilized by the extra-hepatic tissues, it is clear that even such relatively simple foodstuffs do not yield R.Q.'s which represent only the process of oxidation.

Much has been made of the fact that the R.Q. of the whole mammalian organism has not very often been found to fall below 0.7. Indeed it was formerly customary to ascribe any lower R.Q. to some undetected fault in technic. More recently, however, members of the Lusk school have themselves obtained such low R.Q.'s (63) (100) and have reviewed other instances in the literature which are similarly free from technical criticism (100). Some of these low values were obtained in normal human subjects under special conditions of feeding, for example, on high fat intakes before the subjects became acclimatized to the abnormal diet. This is significant because the customary feeding habits of man and of animals have resulted in rather arbitrary conventions as to the number, composition and size of meals, and as to the periods during which R.Q. measurements of the absorptive and post-absorptive states are made. The intake of food is ordinarily spread over a considerable proportion of the 24 hours. This means that all the various oxidations, conversions, etc., which yield the highest and lowest components of the composite R.Q., are usually proceeding simultaneously. Under these circumstances one could hardly expect to obtain anything more than an intermediate range of values for the R.Q. of the whole body.

To succeed in demonstrating a truer range for the component R.Q.'s of the body, on a normal diet, it would be necessary to set the experimental conditions so as to allow the processes responsible for either the lowest or highest component R.Q.'s to predominate temporarily. In other words, it would be necessary "to catch the metabolic processes off balance." This has been done by Werthessen (276) who trained rats to eat their entire 24-hour food requirement within a period of 1 to 5 hours. He found that in the same animal, after such a meal, the R.Q. determined at intervals varied from extremely low to extremely high values. The range of these variations in all his animals was from 0.27 to 1.70! Werthessen concluded that the mechanism of oxidation consists of a series of reactions and that the steps include the conversion

of fat into carbohydrate. Markowitz (personal communication) working with Cathcart, performed this experiment upon himself, and obtained similar results to those reported by Werthessen.

The theoretical R.Q. for the conversion of protein to carbohydrate has been variously calculated as 0.613 (Magnus-Levy—173), 0.632 (Lusk—168) and 0.706 (Geelmuyden—83). The R.Q. for gluconeogenesis from fat has been calculated to be about 0.28 by Pembrey (208) and by Macleod (170). The theoretical R.Q. for ketogenesis from fat may be calculated to range from 0.65 to 0.00, depending upon the number of molecules of β-hydroxybutyric acid which are supposed to arise from one molecule of fatty acid. The work of Blixenkrone-Møller (14) strongly indicates that the value lies closer to zero than to the higher figure. In view of the composite nature of the R.Q. of the whole body and the evidence that carbohydrate is utilized, the diabetic R.Q. of 0.7 can best be interpreted as the resultant of at least two factors, namely, a low component due to gluconeogenesis (especially from fat) and ketogenesis in the liver, and a high component due to the simultaneous oxidation of a portion of the resulting carbohydrate and ketone bodies by the muscles. There are probably many other less influential R.Q.'s, arising from different organs and tissues, which also enter into the balance. But the predominant influence of gluconeogenesis and ketogenesis from fat in lowering the R.Q. is strongly supported by repeated demonstrations that diabetic animals and humans deprived of their fat stores by prolonged under-nutrition yield R.Q. values far above the "diabetic" level (101) (133) (221) (239).

Since gluconeogenesis and ketogenesis occur in the liver, it would be expected that R.Q. determinations performed on the isolated liver under the appropriate physiological conditions should yield very low values. This is the case. Gemmill and Holmes (87) found that the R.Q. of liver slices from a rat fed on a normal diet averaged 0.79, while that from a rat fed butter averaged 0.58. Stadie and co-workers (259b) observed R.Q.s of about 0.32 in liver slices from the depancreatized cat. Similarly in the perfused livers of normal and depancreatized cats, Blixenkrone-Møller (14) obtained R.Q. values which averaged 0.57 for the normal and 0.37 for the diabetic animals. From other data, including the high D:N ratios which he observed, he concluded that the low R.Q. of the diabetic liver resulted from: *a*, the desaturation of fatty acids; *b*, gluconeogenesis from protein and fatty acids; *c*, the formation of ketone bodies. We may summarize by saying that the above con-

siderations of the respiratory quotient show that it in no way contra-indicates carbohydrate utilization in the diabetic organism, or hepatic gluconeogenesis from fatty acids.

d. From the biochemical viewpoint. We have seen that, when the utilization of carbohydrate by the normal and the diabetic organism is not ignored, the conversion of fat to carbohydrate can be indirectly demonstrated. The objections to this conclusion, which are also based on indirect evidence (D:N, ketosis, and R.Q.), have been overcome. There are also a number of experiments which favor gluconeogenesis from fat even though the investigators did not take into account the factor of utilization. In these experiments neutral fat or fatty acids were administered to *intact* normal or diabetic animals; or certain hormones or drugs were given to such animals, in an attempt to force excessive gluconeogenesis from endogenous fat stores. The results of these experiments were judged by the increases in carbohydrate content of the liver and muscles of the normal animals, and by the increased sugar excretion of the diabetic animals. As might be predicted from our previous discussions of the dynamic balance and the D:N ratio, these experiments have yielded both positive (2) (36) (39) (112) (170) (200) (238) (264) and negative (20) (137) (217) results. Under the circumstances it is justifiable to place greater weight on the positive than on the negative findings. Moreover, some of the negative results must be qualified in that sugar was found to be derived from lower fatty acids with an odd number of carbon atoms, but not from lower fatty acids with an even number of carbon atoms (54) (64). It is implied that the naturally occurring even-numbered higher fatty acids may be degraded only by β-oxidation, that only even-numbered lower fatty acids will result, and hence that no sugar can be derived from the naturally occurring fats. As opposed to this it may be pointed out that Jowett and Quastel (136) and Leloir and Muñoz (153), who tested the oxidation products of various fatty acids incubated with isolated tissue slices, concluded that β-oxidation was by no means the only pathway for fatty acid breakdown. In fact, many possible pathways have been demonstrated including α, β, γ, ω, and multiple alternate oxidations (32) (236) (282). Furthermore, whatever the chemical steps by which it proceeds, the conversion of the naturally occurring fats into carbohydrate has been conclusively proven in the germinating seedlings of plants (89) (128) (149) (179) (189) (202) (219) (231). The point at issue, therefore, is not whether the process can occur, but whether it does occur in the mammalian organism.

e. Direct evidence for gluconeogenesis from fat. It remains to consider the more direct evidence concerning gluconeogenesis from fat, when the utilization of carbohydrate by the extra-hepatic tissues is neither affirmed nor denied, but is excluded from the calculation by the experimental method. We have already mentioned the work of Gemmill and Holmes (25) in which they found very low R.Q. values in the isolated liver slices of butter-fed rats. They also observed a coincident increase in the carbohydrate content of these slices, which was greater than the increase observed in liver slices taken from rats on a normal diet. Haarmann and Schroeder (94) (95) added the sodium salts of butyric acid, β-hydroxybutyric acid, and $\alpha\beta$-dihydroxybutyric acid respectively, to surviving tissues (muscle, kidney, spleen, brain and liver) of cats and dogs. With each substance and in practically all tissues they observed a large production of lactic acid. The simultaneous decrease in the carbohydrate content of the tissue, when it occurred, was significantly less than the increase in lactic acid. In the case of the liver, when oxygen was present there was an increase in the carbohydrate content as well as of lactic acid. It was obvious that the lactic acid could not be accounted for as arising from carbohydrate. The authors considered the possibility that the added fatty acids might have stimulated the production of lactic acid from some other substance, but concluded that this supposition could not be justified. They pointed out that in the brain and liver, for example, they were dealing with tissues which ordinarily produce little or no lactic acid, and which contain no other known precursor of lactic acid. Their work, therefore, yields convincing evidence for the formation of carbohydrate from fat through a lactic acid stage. Very recently, gluconeogenesis from fat in isolated mammalian tissue has again been confirmed by Weil-Malherbe (275), who demonstrated the in vitro formation of sugar from added acetoacetic acid by kidney slices.

Another method by which extra-hepatic utilization of sugar has been excluded, and one which is a step nearer the intact organism, is the perfusion of the isolated whole liver. This method is not easy and it is sometimes difficult to obtain satisfactory preparations (91). Nevertheless a number of competent investigators have carried out reasonably successful liver perfusions as judged by a maintained rate of flow of perfusate through the liver with little or no edema, the continued excretion of bile, and the storage of glycogen. Burn and Marks (31) perfused the glycogen-poor livers of fat-fed dogs and of a depancreatized cat. A large production of acetone bodies and of sugar was observed.

The pre-existing carbohydrate content of the livers accounted for but a small fraction of the sugar which appeared. The disappearance of lactic acid was ruled out as a factor. As regards gluconeogenesis from protein, Burn and Marks rightly (in view of our previous discussion of the D:N) rejected the use of any of the orthodox values for the D:N ratio. Instead, they calculated that if all the carbon in the protein molecule were recombined so as to form dextrose, the ratio of dextrose produced to nitrogen set free in the form of urea and ammonia cannot be greater than 8.3:1. Values for the D:N ratio above this figure would therefore demonstrate gluconeogenesis from fatty acid. Out of a total of 47 determinations of the D:N ratio, 32 exceeded the value of 8.3, and in 7 cases the ratio rose above 17.0.

Blixenkrone-Møller (15) perfused the livers of normal and phlorhizinized cats with sodium butyrate. After accounting for other possible sources of carbohydrate, he obtained D:N ratios ranging from 10.0 to 20.0 or over. Perfusion with sodium succinate yielded D:N ratios as high as 42.0. He concluded that 20 per cent of the added butyric acid was converted into ketone bodies and the remainder went to sugar via succinic acid. Finally, Heller devised ingenious methods (102) to observe the sugar output of the liver in situ, in normal and phlorhizinized cats anesthetized with "Pernocton." After deducting the amounts of carbohydrate which might have come from glycogen, lactic acid and glycerol, he calculated D:N ratios ranging from 5.0 to 18.0 (103).

Figure 2 graphically summarizes the more direct evidence for gluconeogenesis from fat, and indicates the intermediate chemical steps by which it may occur. It is concluded that this process can and does play an important rôle in both the normal and diabetic mammalian organism.

PART II. REGULATION OF THE BLOOD SUGAR. *6. The Homeostatic Mechanism in the Liver.* The relative constancy of the blood sugar level in the normal animal, under varied conditions of feeding and fasting, indicates the existence of adequate regulating mechanisms. On the other hand, the hyperglycemia and the great dependence of the blood sugar level of the diabetic organism on the kind and amount of ingested food, indicates a profound disturbance in the regulating mechanisms. Claude Bernard was keenly aware of the dynamic balance involved in carbohydrate metabolism; the balance upon which any proper conception of regulation must be based. He clearly stated that the normal blood sugar level represented a precise equilibrium between the rates

of sugar formation in the liver and sugar utilization in the tissues (88). While the rôle which he assigned to the liver has been confirmed by most

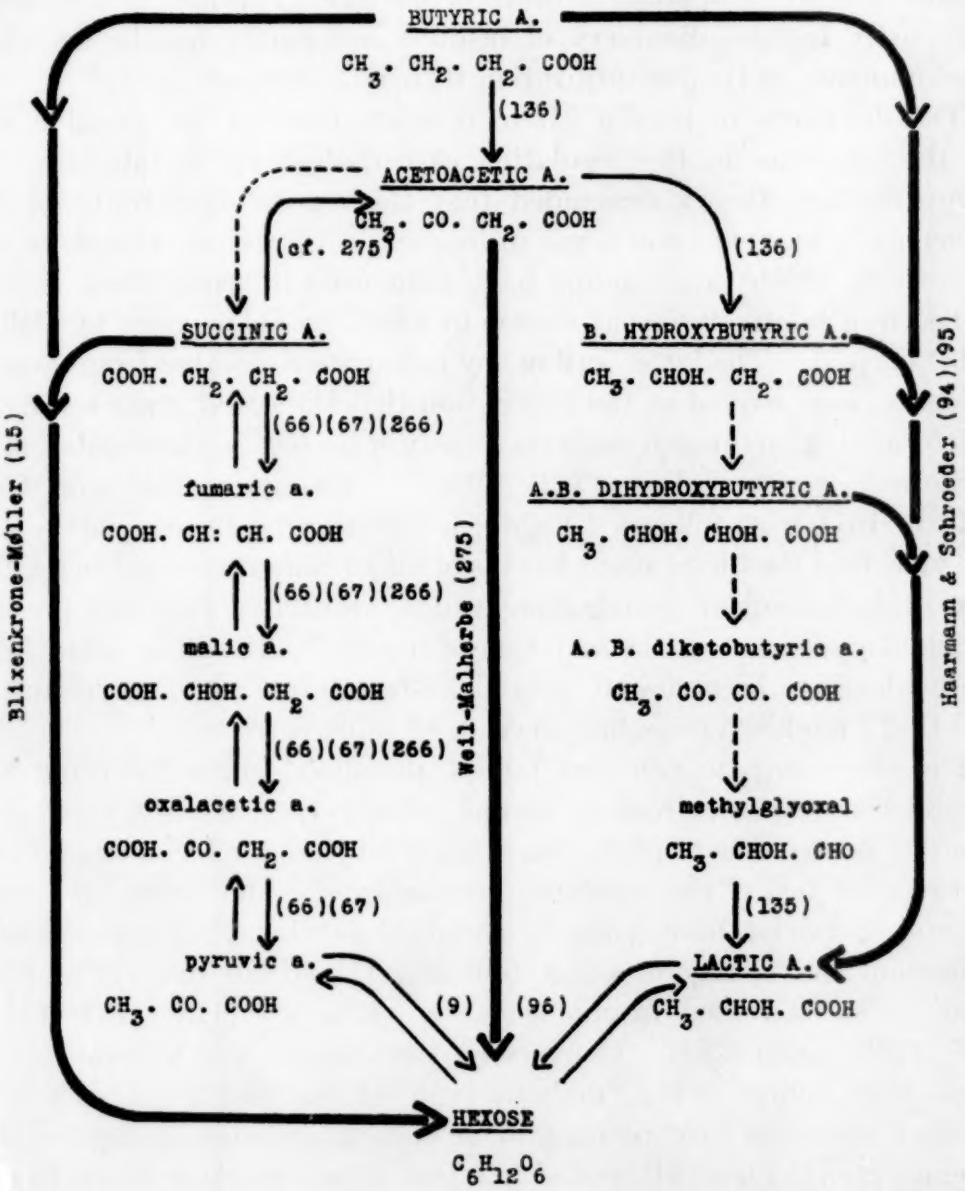


Fig. 2. Gluconeogenesis from fatty acid. The heavy continuous lines indicate the beginning and end products in the direct demonstrations of gluconeogenesis. The light continuous lines indicate the demonstrated intermediate reactions by which gluconeogenesis may proceed. The light broken lines indicate intermediate reactions which have not been directly demonstrated. The bracketted numbers are references to the literature on the respective reactions.

recent workers (41) (78) (88) (142) (143) (158) (267) (280) it has nevertheless been virtually ignored in the usual explanations of the various

experimental or clinical states which are characterized by a persistence of abnormal blood sugar levels. Instead, attention has been focussed almost exclusively upon the utilization of sugar. This may be accounted for partly by the discovery of insulin, and partly by the erstwhile predominance of the non-utilization theory of diabetes.

The discovery of insulin led to over-emphasis of the possible rôle of the pancreas in the regulation of carbohydrate metabolism; the non-utilization theory demanded that the regulating activity of the pancreas be exerted upon sugar utilization. A striking example of the manner in which these factors have influenced interpretations is contained in a relatively recent review in which an older paper by Pollak (213) is cited. The latter author, by fortunate deduction from meagre evidence, had arrived at the conception that the blood sugar level was a determining factor *as regards the activity of the liver* in the regulation of carbohydrate metabolism (212) (213). The quotation from Cori (42—p. 197) is as follows: "Pollak, before the insulin era, advocated the view that the blood sugar level is of major importance in the regulation of carbohydrate metabolism, which, translated into our present terminology, means in the secretion of insulin." It will be seen, from the evidence to be reviewed, that this "translation" is not warranted, and that Pollak's version happened to be more correct.

The characteristic rise and fall of the blood sugar following the administration of dextrose to normal animals, represents a rapid and accurate re-adjustment of the blood sugar level, and offers a simple and reproducible test of the regulating mechanisms. This normal dextrose tolerance curve has been generally explained as being due to an increased utilization and storage of sugar, following the stimulation of the pancreas to an increased insulin secretion (42) (55) (113) (132) (141) (205) (260) (261) (281). Conversely, the sluggish and incomplete re-adjustment known as the "diabetic type" of dextrose tolerance curve, whether occurring in experimental or clinical diabetes, starvation, or toxemia, etc., has been attributed to a lack of pancreatic response to the administered sugar, and a consequent deficiency in sugar utilization and storage (52) (55) (90) (176) (260) (265) (281).

More recently, Soskin and co-workers (243) tested the fundamental basis of these explanations by substituting a constant injection pump for the pancreas, as the source of insulin in dogs. These depancreatized animals, while receiving a steady supply of insulin just sufficient to maintain a constant blood sugar level, differed from normal animals in

that they could not mobilize an additional supply of insulin when sugar was administered. Nevertheless, they yielded normal dextrose tolerance tests, including the Staub-Traugott phenomenon and the hypoglycemic phase (242). But, when hepatectomized dogs, with pancreas intact, were maintained by a constant injection of dextrose just sufficient to keep them at a normal blood sugar level, they invariably yielded markedly diabetic dextrose tolerance curves. It was apparent that the pancreas was not essential to the regulating mechanisms responsible for the normal dextrose tolerance curve, while the presence of the normal liver was essential. This led to observations on the simultaneous blood sugar values of the blood flowing into and out of the liver, in normal and depancreatized dogs, during the course of dextrose tolerance tests. From these and the previous results, it was postulated that, in the presence of a sufficiency of insulin, but not necessarily an extra secretion from the pancreas, the normal liver, as one of its responses to administered dextrose, decreases the output of blood sugar which it has been previously supplying from its own resources. This homeostatic mechanism was later directly proven by Soskin, Essex, Herrick and Mann (246), using blood sugar values and thermostromuhr measurements of bloodflow to calculate the quantitative output and intake of sugar by the intact liver in situ.

The former conception of dextrose tolerance, as compared to the hepatic mechanism which has been demonstrated, is analogous to the difference between an old-fashioned stove and a thermostatically controlled furnace. In the former any excess over the heat requirements is wasted at the expense of extra fuel consumption. In the latter, the rise in temperature above the threshold of the thermostat shuts off the furnace and conserves the fuel until the temperature falls to a level somewhat below the threshold, whereupon the furnace again becomes active. In this analogy, the temperature is equivalent to the blood sugar level and the thermostat-furnace arrangement is the liver. Accordingly, the dextrose tolerance curve and the hypoglycemic phase which often follows it, resemble the fluctuations in temperature above and below the threshold of regulation when an extra quantity of heat is introduced into the system. The characteristics of the curve depend upon the magnitude of the disturbing factor (the amount of sugar administered), the setting and sensitivity of the thermostat (the endocrine balance), and the capacity of the furnace (the ability of the liver to produce sugar).

From this point of view insulin is merely one of the factors which

determine the threshold of the homeostatic mechanism, and the amount of insulin need not vary to insure adequate regulation. In the normal animal, a rise in the blood sugar level causes the liver to respond by diminishing its output of sugar to the blood. A coincident decrease in gluconeogenesis, by removing a low component of the composite R.Q., can explain part of the rise in the R.Q. which follows sugar administration. The stimulus which elicits the hepatic inhibitory response is the blood sugar itself, and the threshold of stimulation of the hepatic mechanism in a particular animal depends largely upon the endocrine balance, and coincides with the level of blood sugar which that animal habitually maintains.

Although other glands are undoubtedly involved, it may be supposed for the sake of simplicity that the endocrine balance consists of the opposing influences of the hormones of the pancreas and of the anterior hypophysis. When insulin is lacking or when anterior pituitary hormone is present in excess, the liver threshold rises and hyperglycemia results. An excess of insulin or a deficiency of the anterior pituitary hormone lowers the liver threshold so that the pre-existing blood sugar level becomes an adequate stimulus to inhibit the liver, and hypoglycemia occurs. When both insulin and the anterior hypophysis are lacking, as in the "Houssay" animal, the diabetes is ameliorated, and it is possible to obtain most of the criteria of normal carbohydrate metabolism (116) (256), *including normal dextrose tolerance curves* (257), at a glycemic level in between the usual blood sugar levels of hypophysectomized and depancreatized animals. In the depancreatized animal with hypophysis intact, the hyperglycemias caused by sugar administration do not reach the infinitely high threshold of the diabetic liver, which therefore is not inhibited and continues to form and pour sugar into the blood. This lack of hepatic inhibitory effect accounts for the difficulty in influencing the diabetic R.Q. and for the so-called quantitative return of the administered sugar. Thus the metabolic disturbance in diabetes regarded from the standpoint of blood sugar regulation, coincides with the "Overproduction Theory" which we have already been forced to adopt on the basis of the other evidence which we have evaluated. From the same standpoint, other disturbances of carbohydrate metabolism such as those occurring in toxemia (48) (244) (254) are also more readily understood.

The fact that the hepatectomized animal with an artificially maintained normal constant blood sugar level (and with the pancreas and extra-hepatic tissues free to exert whatever regulating powers they

possess) yields "diabetic" dextrose tolerance curves (243),[1] indicates the essential rôle of the liver in this regard. It is not to be supposed, however, that the hepatic mechanism is the only one involved. Glycogen deposition in both the liver and muscle, and an increased utilization of sugar by the extra-hepatic tissues, undoubtedly play their parts. These processes, like hepatic homeostasis, are under the influence of the blood sugar level. Cori and Cori (44) have pointed out that the rate of glycogen deposition depends upon the concentration of sugar in the blood. Soskin and Levine (247) have shown that the rate of sugar

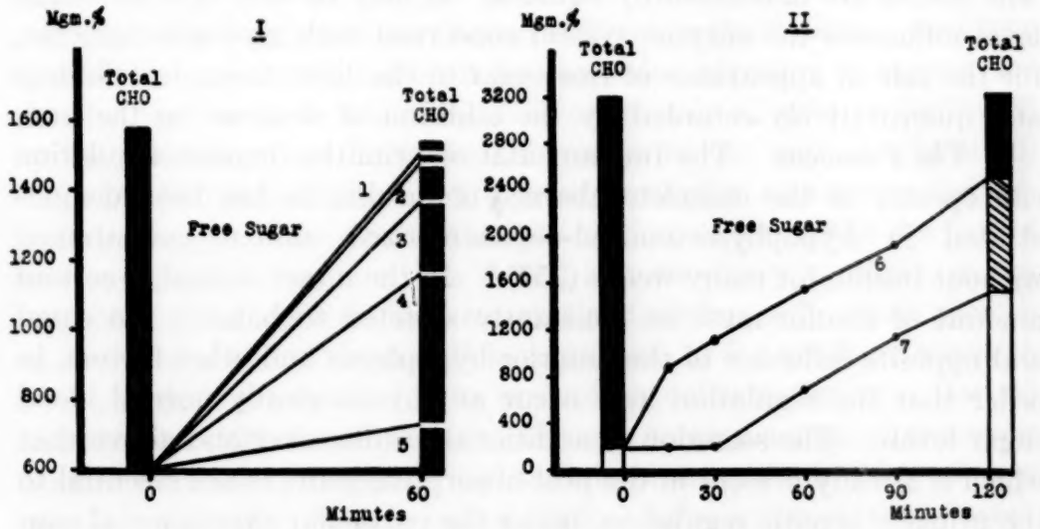


Fig. 3. I. The influence of different amounts of dextrose added to each vessel, upon the appearance of free sugar in liver brei in 1 hour. *1*, no addition; *2*, 5 mgm. dextrose added; *3*, 10 mgm. added; *4*, 20 mgm. added; *5*, 40 mgm. added.

II. A comparison of the rates of appearance of free sugar at different time intervals, with and without the addition of dextrose to liver brei. *6*, no addition; *7*, 20 mgm. dextrose added. The blocks representing total carbohydrate determinations at the beginning and end of each experiment indicate that there was no significant loss of carbohydrate from the system.

utilization by the extra-hepatic tissues varies directly with the height of the blood sugar level. It seems logical to assume that smaller amounts of sugar, especially if they enter the circulation via the portal vein, may be fully compensated for by hepatic inhibition alone. Larger

[1] The liverless animal in which a constant blood sugar level is not being maintained, will exhibit a curve resembling a normal dextrose tolerance test when given an amount of sugar which its tissues are capable of utilizing within 2 hours, namely, 0.5 gram or less per kilogram of body weight. But larger amounts of sugar yield "diabetic" looking curves, showing that the results obtained have little relation to dextrose tolerance as far as regulating mechanisms are concerned.

amounts of sugar will invoke hepatic storage as well. Still larger amounts, which in spite of the above raise the systemic blood sugar level, will bring into play the additional factors of extra-hepatic storage and increased utilization.

It is clear that the fundamental regulation of the blood sugar is an auto-regulation, in which the prime mover is the blood sugar level itself. This is further supported by the work of Soskin, Levine and Taubenhaus (253) on the rate of appearance of free sugar in glycogenolyzing liver brei, with and without the presence of added dextrose. The results are illustrated by figure 3. It may be seen that the sugar level influences the enzyme system concerned with glycogen⇄glucose, for the rate of appearance of free sugar in the liver tissue is definitely and quantitatively retarded by the addition of dextrose to the brei.

7. The Pancreas. The fundamental or primitive hepatic regulation can operate in the complete absence of insulin, as has been demonstrated in hypophysectomized-depancreatized animals maintained without insulin for many weeks (257).[2] In the intact animal, a certain amount of insulin must be constantly secreted to balance the equal and opposite influence of the anterior hypophysis and other factors, in order that the regulation may occur at physiologically normal blood sugar levels. The secretion of additional insulin, over and above that which is already present in the post-absorptive state, is not essential to the primary hepatic regulation, under the particular experimental conditions in which this has been tested (242) (243). However, this does not contradict the considerable body of evidence which indicates that extra insulin is ordinarily secreted as a result of hyperglycemia (82) (99) (124) (125) (147) (148) (262) (296) (297). But it is not entirely certain what purposes are served by this additional insulin. Ricketts (223) has reported that 5 diabetic humans and 2 completely depancreatized dogs, given a dose of protamine insulin on the preceding evening sufficient to maintain their blood sugar at normal levels throughout the following morning if no food was taken, nevertheless yielded diabetic tolerance curves in response to breakfast. On the other hand, Pollack and Dolger (211) performed glucose tolerance tests on 52 diabetic patients, who had received their last dose of protamine zinc insulin 24 hours previously, and obtained normal tolerance curves in many

[2] This effectively disposes of Himsworth's hypothesis (104) (105) according to which the hyperglycemia which follows dextrose administration causes a liberation of "insulin kinase" from the liver, which in turn activates the previously *inert* insulin already present in the blood stream.

instances. Soskin and Hyman, in as yet unpublished experiments closely patterned after those of Ricketts, obtained results similar to those reported by Pollack and Dolger. It seems fair to conclude that, while the secretion of extra insulin is not essential to the intrinsic hepatic mechanism, it acts under normal physiological conditions as a factor of safety in increasing the efficiency of this type of regulation.

As regards the other effects of the provoked extra supply of insulin, it tends to decrease carbohydrate storage in the liver of the normal animal, rather than to increase it (6) (18) (44) (218). It does not cause any increase in the rate of sugar utilization by the extra-hepatic tissues, over that which results from the hyperglycemia itself (248). One way in which it can aid regulation is by increasing glycogen storage in the muscles, for this is one function which practically all agree is facilitated by the administration of additional insulin to the normal animal (6) (12) (13) (43) (45) (248). Extra insulin probably also serves the secondary purpose of decreasing the rate of gluconeogenesis in the face of an oversupply of sugar. This action is a necessary corollary to our consideration of diabetes as a metabolic disturbance in which there is an overproduction of sugar. It is supported, as far as gluconeogenesis from protein is concerned, by the work of several investigators. Janney and Shapiro (129) found that the nitrogen sparing action of glucose in normal animals is increased by the simultaneous administration of insulin. Similarly, MacKay et al. (168a) have reported that, in rats maintained on a diet containing 20 per cent casein and 41 per cent starch, the administration of insulin caused the nitrogen balance to become more positive, whether the food intake was fixed in amount or was given ad lib. Bach and Holmes (4) showed that insulin decreased the gluconeogenesis observed in excised liver slices in vitro, and that this diminution was accompanied by a depression of urea formation. Stadie et al. (259a) have confirmed the fact that insulin inhibits the in vitro oxidative deamination of amino-acids, although they found that the inhibition was not exerted on the l-amino acids, and occurred consistently with the d-amino acids. Mirsky and associates (194) (201) demonstrated that insulin caused a definite decrease in the mobilization of amino-acids from the muscles, probably by stimulating the synthesis of muscle protein. The lowering of the blood amino-acids by insulin has also been confirmed by others (47a) (75a).

8. The Anterior Hypophysis. The secretion of the anterior lobe of the pituitary gland opposes the action of insulin in the regulation of the blood sugar. Its opposition, as far as the liver is concerned, is mainly

focussed where insulin exerts its chief effects, namely, on the threshold of regulation of the homeostatic mechanism, and on the rate of gluconeogenesis. After removal of the hypophysis from normal or depancreatized dogs, *well-fed* animals maintain significantly lower blood sugar levels than before hypophysectomy. This is due to the influence of the hypophysis on the threshold of the homeostatic mechanism, which has already been discussed (257). The *fasting* hypophysectomized animal, even when depancreatized, shows a progressive drop in the blood sugar level, and may die in hypoglycemic convulsions (115) (116) (166) (174) (256). There is good evidence that the hypoglycemia of fasting after hypophysectomy is due to a decreased rate of sugar formation by the liver.

The decreased nitrogen excretion of hypophysectomized dogs, and the low D:N ratios exhibited by hypophysectomized-depancreatized animals led Houssay (114) (116), Long and others (160) (165) to conclude that hypophysectomy decreases gluconeogenesis from protein. On the other hand, Soskin et al. (256) showed that meat-fed hypophysectomized dogs had no difficulty in sustaining their blood sugar levels at the expense of the amino-acids derived from the gastro-intestinal tract. But when such animals were fasted, they were unable to catabolize their endogenous body proteins to amino-acids rapidly enough to support a gluconeogenesis sufficient to maintain their blood sugar levels (250). Since orally administered fat did not maintain the blood sugar level of fasting hypophysectomized dogs (256), it was concluded that such animals suffered from a disability to derive an adequate blood sugar supply from both protein and fatty acid, while fed hypophysectomized animals were limited to ingested protein as their chief non-carbohydrate source of blood sugar. The relation of the anterior pituitary to gluconeogenesis from fatty acid is also suggested by the recent work of Neufeld, Scoggan and Stewart (204a). They injected various anterior pituitary extracts as prepared in Collip's laboratory into female mice, and made chemical determinations of the entire carcass of their animals. They found an increase in the total glycogen content, a decrease in the amount of fatty acids present and no change in the nitrogen.

On the other hand, Fisher, Russel and Cori and others concluded that the effects of hypophysectomy resulted from a relatively greater oxidation of carbohydrate (76) (77) (227) and that the administration of pituitary extracts inhibits the utilization of carbohydrate by fixing the body glycogen (the so-called "glycostatic effect") (227) (228) (229). Their conclusions were based upon the calculation of the amounts of

carbohydrate oxidized from R.Q. estimations, and upon carbohydrate balance experiments performed on *intact* fasting animals. We have already discussed the difficulty of accepting quantitative deductions concerning the oxidation of foodstuffs based on R.Q. determinations in the whole animal. Similarly, carbohydrate balance studies done on the intact animal ignore the dynamic balance to which we have referred, for they do not take into account the unknown amounts of sugar formed by the liver during the experimental period.

Accordingly, Soskin and co-workers (249) (252) obtained direct data on the rate of sugar consumption of the extra-hepatic tissues in hypophysectomized dogs, and in normal dogs treated with an active anterior pituitary extract. The variable factor of hepatic gluconeogenesis was eliminated by substituting for it a constant injection of known amounts of dextrose, in abdominally eviscerated dogs. It was found that the extra-hepatic tissues of hypophysectomized dogs utilized sugar at subnormal rates, and that the pituitary extract (which was shown to be capable of maintaining the carbohydrate levels of fasting, hypophysectomized animals) did not influence the rate of sugar utilization by the extra-hepatic tissues of normal dogs. It is therefore, evident that the decrease in carbohydrate levels exhibited by fasting hypophysectomized animals is due to a decreased rate of hepatic gluconeogenesis, insufficient to meet even the reduced sugar utilization of the extra-hepatic tissues. Conversely, the increased carbohydrate levels resulting from the administration of anterior pituitary extracts ("glycostatic effect") is not due to a greater stability of the tissue glycogen, but results from the stimulation of hepatic gluconeogenesis to a rate in excess of the sugar utilization of the extra-hepatic tissues. Crandall and Cherry (46) have confirmed the influence of the hypophysis on hepatic gluconeogenesis in intact non-anesthetized normal and hypophysectomized animals, by means of the London cannula technique. From the blood sugar contents of the inflowing and outflowing hepatic blood, they estimated that the rate of sugar output from the livers of their fasting hypophysectomized dogs was only about 50 per cent of the output from the livers of fasting normal dogs.

The internal secretions of the pancreas and anterior hypophysis are the major opposing forces in the endocrine balance affecting the regulation of the blood sugar level. The thyroid gland and adrenal cortex may be regarded as subsidiary forces, controlled by the pituitary, and acting in the same direction.

9. The Thyroid. Clinicians have long recognized the influence of

hyperthyroid or hypothyroid states on carbohydrate tolerance (131) (187) and on co-existing diabetes mellitus in humans (134) (226). In sheep Bodansky (16) found that thyroidectomy caused a decrease in the blood sugar level, while thyroxin administration raised it in normal as well as in thyroidectomized animals. However, since thyroidectomy of the normal or depancreatized dog and cat apparently had little influence on their carbohydrate tolerance, many writers have been led to minimize the rôle of the thyroid in this regard (160) (283) (294). Recently Dohan and Lukens (58) re-investigated the effect of thyroidectomy upon pancreatic diabetes in the cat. The small (though significant) influence which they observed, as compared to the marked effects of hypophysectomy, led them to conclude that the secondary atrophy of the thyroid gland plays a relatively small part in the profound modification of diabetes which follows removal of the hypophysis from the depancreatized animal. It seems probable, however, that the results obtained after thyroidectomy in the common laboratory animals are invalid because the animals do not become really hypothyroid. Marine (180) has demonstrated aberrant thyroid tissue in over 90 per cent of these animals.

Accordingly, Soskin and co-workers (250) studied the problem by administering thyroxin to hypophysectomized dogs. They found that the thyroid hormone maintained the blood sugar level of fasting hypophysectomized dogs not merely at levels which usually occur in the fed hypophysectomized animal, but at levels which are characteristic of the normal dog. This effect could not be ascribed to a further decrease in the already low rate of utilization of sugar by the extra-hepatic tissues of the hypophysectomized dog, for there is good evidence that the thyroid increases the utilization of sugar by the peripheral tissues (92) (186) (195) (215). Thyroxin also increased the urinary nitrogen excretion of the fasting hypophysectomized dogs to that of fasting normal dogs. It was, therefore, concluded that the secondary atrophy of the thyroid gland is partly responsible for the decreased gluconeogenesis which follows hypophysectomy, probably by decreasing endogenous protein → amino-acid catabolism. The influence of the thyroid on gluconeogenesis from protein has been confirmed more recently by Sternheimer (263a) and by Wells et al. (275a) (275b) (275c). The in vitro observations of Klein (140a) of an increased d-amino acid oxidase activity in the liver slices of thyroid-fed rats, also suggest that the effects of thyroid are not confined to the breakdown of protein above the amino acid stage. Part of the confusion which has arisen from the

minor effects of thyroidectomy of (58) and of thyroid administration to (166a) depancreatized animals, may depend upon the possibility that the presence of the internal secretions of the pancreas is essential for the gluconeogenetic effects of the thyroid hormone (241a).

10. The Adrenal Cortex. Whether the adrenal cortex influences carbohydrate metabolism primarily (24) (25) (26) (28) (234) or whether its effects are secondary to its control of electrolyte balance (27) (79) (97) (140) (235), is not pertinent to the present discussion. Suffice it to say that total adrenalectomy causes a lowered blood sugar level and decreases all carbohydrate stores in the body (24) (26) (28) (234). It seems likely that the adrenal cortex affects carbohydrate metabolism both directly and indirectly (241a) for, while glucose absorption (1a) and glycogen deposition (1b) may occur normally in salt-treated adrenalectomized rats, nevertheless when such animals are fasted they suffer a sharp decline in the carbohydrate levels of all tissues despite salt treatment (75b) (162a). The lack of adrenal cortical hormone also results in an amelioration of pancreatic diabetes in dogs (163), cats (98) (160) (163) (164) and toads (117), and of diabetes mellitus in the human (15a). These effects are not due to the absence of the adrenal medulla (29) (122) (224) (268) (295). Long, Fry and Thompson (161) (162a) demonstrated that the glycosuria of partially depancreatized rats, which disappears after adrenalectomy, can be made to reappear by the administration of large amounts of a purified adrenal cortical extract. Similar results have been obtained by others (15a) (126a). But, what is perhaps more significant, this same extract increased the liver glycogen in normal rats (138).

Long and Katzin (162) and Russell and Craig (230) have shown that adequate treatment with adrenal cortical hormone prevents the lowering of carbohydrate levels in fasting hypophysectomized rats, or restores them after they have been decreased. The blood sugar and liver glycogen values of the treated rats may exceed the values found in fasting normal rats. However, the adrenal cortex cannot substitute completely for the anterior hypophysis, nor is it essential to all the activities of the latter. Bennett (8) has found that adrenal cortical hormone plus sodium chloride, while maintaining normal muscle glycogen values in fasting adrenalectomized rats, will not do so in hypophysectomized rats. He also showed that, even after complete adrenalectomy, anterior pituitary extract still maintained the muscle glycogen levels of fasting hypophysectomized rats. That the adrenal cortex is not essential for the action of the anterior hypophysis on the

liver is clear from the work of Houssay and Leloir (121) who were able to maintain the diabetic state by hypophyseal extract administration in adrenalectomized dogs. It thus appears that the adrenal cortex stimulates hepatic gluconeogenesis, but is not a necessary intermediary for the anterior hypophysis in this respect. It is possible that the latter stimulates gluconeogenesis from one precursor either directly or through the thyroid gland, and stimulates sugar formation from another precursor through the adrenal cortex.

From the fact that, in some experiments (73) (74) (160), variations in carbohydrate formation caused by experimental variations in adrenal cortical activity have been found to be accompanied by changes in nitrogen excretion, it has been concluded that the adrenal cortex is concerned with gluconeogenesis from protein. There is other work, however, which indicates that the adrenal cortex may also affect gluconeogenesis from fat. Verzar and co-workers (269) (270) (272) have shown that adrenalectomy interferes with the absorption of fat from the gastro-intestinal tract, because of a disturbance in the phosphorylating mechanisms, and have concluded that the adrenal cortex is primarily concerned with phosphate transfer. Recent attempts to confirm these findings and conclusions have been almost uniformly unsuccessful (29a) (41a) (41b) (206a) (226a). However, it seems certain that the adrenalectomized animal suffers from a disability as regards the mobilization of fat from the depots and its transport to the liver. The phospholipids and fatty acids of the blood are decreased after adrenalectomy (286), and various procedures which ordinarily increase the fat content of the liver no longer do so (6a) (169) (271). In addition, Hochfeld (112) has shown that the livers of fat-fed rats treated with adrenal cortical hormone exhibit very significant increases in glycogen, as compared to the livers of untreated fat-fed rats or those of treated rats on a mixed diet. From the standpoint of the dynamic balance, the evaluation of the above evidence as regards the influence of the adrenal cortex on hepatic gluconeogenesis, whether from protein or fatty acids, must await knowledge as to the possible effect of this gland on sugar utilization by the extra-hepatic tissues. It seems likely that peripheral utilization is not an important factor, for Selye and Dosne (231a) have found that while cortin will inhibit the fall in blood sugar of partially hepatectomized rats, it fails to have any effect in completely liverless animals.

11. The Unknown Factors in Regulation. Thus far, our account of the regulation of the blood sugar has dealt chiefly with a biochemical

system in the liver, the equilibrium of which is governed by the opposing forces of the pancreas on the one hand, and of the anterior pituitary, thyroid and adrenal cortex, on the other. It has been pointed out that the endocrine glands also affect the activity of the extra-hepatic tissues, although the results of these peripheral actions are apparently not as important to blood sugar regulation as their actions on the liver. However, to say that we have now explained the regulation of the blood sugar level would be an absurd oversimplification of the problem. It is evident, for instance, that the mechanisms which have been discussed do not explain how the endocrine balance is established at the particular height which results in the normal blood sugar level of a particular animal or species of animal. Soskin and Levine (247) have shown that the hyperglycemia of the depancreatized dog enables its extra-hepatic tissues to utilize as much sugar, as the tissues of the normal animal utilize at the normal blood sugar level. But, unless one adopts the purely teleological reasoning of Himsworth (106), it is insufficient merely to state that the height of the blood sugar level is adjusted according to the circumstantial needs of the tissues which utilize sugar. How are these needs communicated to the regulating system? What governs the plane of activity of each endocrine gland? Only fragmentary answers are available. For example, Gerard and McIntyre (87a) working with the thyroid, and others with other glands, have shown that the level of a given hormone in the blood may have an important regulating activity upon the gland which secretes that hormone. It is also possible that the blood sugar level itself plays as important a part in these connections as it does in the homeostatic mechanism of the liver. Himsworth and Scott (107) have compared the effects of a low carbohydrate diet on sugar tolerance and insulin sensitivity with the results of anterior pituitary extract administration, and have ascribed the former to an increased functional activity of the anterior hypophysis. Soskin et al. (258) have reported that the increasing carbohydrate tolerance exhibited by the normal animal which is receiving repeated administrations of dextrose (Staub-Traugott phenomenon), may be explained by a gradual depression of the functional activity of the anterior hypophysis under the influence of a high carbohydrate environment.

It is also possible that the various endocrine glands exert mutually regulatory effects directly on each other, through their internal secretions. This might explain the increased sensitivity of experimental animals to the administration of hormones or glandular extracts, after

ablation of those glands which exert an opposing influence. Perhaps a more striking example of the influence of one gland on another is contained in the work of Young, who climaxed the previous demonstrations of the diabetogenic effect of pituitary extracts (7) (75) (118) by producing permanent diabetes in dogs by the administration of increasing, massive doses of extract (290) (291) (292) (293). At first glance it appeared that he had produced a permanent diabetic state which differed from pancreatic diabetes. It was soon shown, however, that the permanent diabetes of Young was accompanied by an extreme degeneration of the islets of Langerhans of the pancreas (33) (59) (222). It seems likely that the presence of excessive amounts of anterior pituitary hormones in Young's dogs, evoked a correspondingly excessive secretion of insulin, which eventually resulted in an overwork atrophy of the islet cells (10a) (96a) (221a) (241a).

12. The Emergency Mechanisms. Finally, it is necessary to mention the parts played by the central and autonomic nervous systems and by the adrenal medulla, in the control of the blood sugar level. These agencies may be regarded as emergency safeguards against blood sugar variations, particularly hypoglycemia, too rapid or too severe to be adequately handled by the regulating mechanisms proper which have already been discussed. It is beyond the scope of this review to discuss these emergency mechanisms in detail. It may be pointed out, however, that their peculiar status is revealed by the fact that adequate regulation of the blood sugar level (except for an increased sensitivity to insulin) ordinarily persists, even after all possible influence of the emergency mechanisms has been eliminated. This has been shown after denervation of the liver (60), denervation or grafting of the pancreas (1) (80) (81) (123) (124) (125) (126) (157), denervation or destruction of the adrenal medulla (23) (29) (152), bilateral vagotomy (210) (214) and total sympathectomy (29) (62). It may be helpful to think of the relationships between the emergency mechanisms, the endocrine glands, and the intrinsic hepatic homeostasis, from the phylogenetic viewpoint. The fundamental or primitive regulation may be supposed to reside in the biochemical processes of the tissue cells. The endocrine glands may represent a step up the evolutionary scale by providing a more sensitive and finely adjusted regulating mechanism, which renders the more highly developed organism less dependent upon its external environment. The emergency mechanisms may be an additional protection against hypoglycemia, for the highly specialized tissues (e.g., central nervous system) of the most highly developed organisms.

PART III. UTILIZATION OF THE BLOOD SUGAR. *13. Significance of "Utilization" Versus "Oxidation."* The term "utilization" is employed throughout this Review, except in the sections relating to the respiratory quotient. It is used to denote the disappearance of sugar from the blood and extra-hepatic tissues, to produce useful energy and heat. The storage of carbohydrate or its return to the bloodstream in altered form are not included within the meaning of the term. The word "oxidation" has been avoided wherever possible, because it carries with it certain traditional physiologic connotations which are no longer acceptable in the light of present-day biochemistry (266). Chief amongst these is the old conception that, by the simple addition of oxygen to their molecules within the tissues of the effector organs, the original foodstuffs may liberate their energy by complete oxidation to carbon dioxide and water. Since the respiratory quotient purports to be a qualitative and quantitative index of this type of reaction, the term "oxidation" is perforce used in connection with it. But, as will appear from the evidence to be reviewed, the oxidative breakdown of the energy materials in the tissues is actually a far more complicated matter, into which the respiratory quotient can give little insight. For this reason, it is hardly more justifiable to speak of "oxidation" as judged from the R.Q., than it is to use the same term to describe the simple disappearance of a substance as determined by chemical balance studies. It would be more accurate to employ the term "utilization" as we have done, and to reserve the term "oxidation" for those rather exceptional instances where the various intermediary metabolites in the degradation of a foodstuff have been chemically identified as oxidation products.

The previous discussion of the respiratory quotient (in relation to gluconeogenesis from fat) allowed the basic postulates of the R.Q. to pass unchallenged, for the time being. In the customary language associated with the subject, it was shown that the R.Q. of the whole body is a composite of many different R.Q.'s arising in the various organs and tissues of the organism. It was pointed out that these individual R.Q.'s were derived from multiple interconversions as well as from purely catabolic oxidations, and it was concluded that the total or average R.Q. could not represent only the kind and amount of foodstuff being oxidized. This suggests that, although the classical physiological interpretation of the R.Q. of the whole animal cannot be upheld, it might be valid if applied to individual organs or tissues. The following discussion of the possible significance of the respiratory quotient, is therefore based upon the evidence obtained under the simplified con-

ditions made possible by the Warburg technique for the study of the respiration of isolated tissues.

14. Significance of the R.Q. as Judged from In Vitro Determinations on Isolated Tissues. Under the simplest conditions, a single known substrate can be exposed to a single isolated enzyme system. If the reaction which follows is known to proceed to carbon dioxide and water, without the formation of stable intermediate substances, the amount of substrate which has been oxidized can readily be computed from the oxygen consumed or from the carbon dioxide produced. If a stable intermediate substance of known chemical composition is formed, the R.Q. may be used to calculate the course of the reaction (159). However, it is usually also necessary to determine the amount of original substrate which has disappeared, or the amount of intermediate substance which has appeared, by chemical analysis. When a single substrate is acted upon by an enzyme system and an unknown stable intermediate substance is formed, the difference between the theoretical R.Q. for the complete oxidation of the substrate and the actual R.Q. obtained, may suggest the probable identity of the unknown intermediate (159).

There is no tissue which does not contain a number of substrates and more than one enzyme system. In working with a tissue it is therefore desirable to allow it to approach the zero level of auto-respiration (i.e., to exhaust its own substrates) before the substrate under investigation is added. If the R.Q. of the subsequent reaction agrees with the chemical determination of the disappearance of the added substrate and the appearance of end products, it may then be concluded that the particular enzyme system which it was hoped to engage has operated, and that the supposed course of the oxidative process has been confirmed. It is thus apparent that, even when one can control the other activities of an isolated tissue and is dealing with a single substrate, the R.Q. is merely confirmatory to the information obtained by chemical analysis. When used alone the R.Q. can, at most, merely suggest the probable pathway of a reaction, which must then be demonstrated by chemical means. To illustrate the lack of preciseness of the indications derived from the R.Q., let us suppose that the substrate is hexose, and that no other foodstuff is involved. Let us simplify matters further by considering the possible pathways open to just one of its important intermediary metabolites, namely, pyruvic acid.

Table 3 summarizes the rather formidable list of possibilities, with the experimental or theoretical R.Q. of each. The various observed

total R.Q.'s for pyruvic acid which are cited, have been obtained in different tissues and circumstances, and depend upon the particular combination of the individual reactions favored by the experimental conditions. It is obvious that the total R.Q. of a single tissue, like that of the whole body, is a composite of many possible R.Q.'s. It is also clear that to gain more than the vaguest indication of the fate of the substrate from the R.Q. alone, is a mathematical impossibility. Furthermore, when the chemical determinations have been made, there is little information that the total R.Q. can add, except to act as a check on the possibility that one or more of the end products might have been missed.

TABLE 3

Possible pathways of pyruvate metabolism

REFERENCES	REACTION PRODUCTS	O_2 CONSUMED	CO_2 PRODUCED	THEORETICAL R. Q.
		Mm/M. pyruvate	*Mm/M. pyruvate*	
Braunstein and Kritzman (21)	$CH_3CHNH_2 \cdot COOH$ (Alanine)	0	0	$\frac{0}{0} = 0$
Benoy and Elliott (9)	$C_6H_{12}O_6$ (Hexose)	−0.5	0.0	$\frac{0}{-0.5} = 0$
Long (159)	CO_2+H_2O	2.5	3.0	$\frac{3}{2.5} = 1.2$
Elliott and Greig (66). Weil-Malherbe (274). Krebs and Johnson (145)	$COOH \cdot CH_2CH_2 \cdot COOH$ (Succinic A)	0.75	1.0	$\frac{1.0}{0.75} = 1.33$
Krebs and Johnson (146) (145)	CH_3COCH_2COOH (Acetoacetic A)	0.5	1.0	$\frac{1}{0.5} = 2$
Long (159)	$CH_3COOH+CO_2$ (Acetic A)	0.5	1.0	$\frac{1}{0.5} = 2$
Krebs and Johnson (145)	$CH_3COOH+CH_3CHOHCOOH+CO_2$ (Acetic A) (Lactic A)	0	0.5	$\frac{0.5}{0} = 0$

Observed RQs of pyruvate in various tissues

REFERENCES	TISSUE	OBSERVED R. Q.
Bach and Holmes (4)	Liver	0.82–1.11
Elliott and Schroeder (68)	Kidney	1.07–1.24
Elliott, Greig and Benoy (67)	Testis	1.17–1.41
Elliott, Greig and Benoy (67)	Brain	1.18–1.28
Long (159)	Brain	1.28
Elliott, Greig and Benoy (67)	Liver	1.19–1.76

If we now attempt to apply the above to the intepretation of the R.Q. in vivo, there is one further complication which must be mentioned. In the body, the three main foodstuffs or their break-down products

are constantly available, and may be metabolizing simultaneously. It has been shown that amino-acids may yield the same R.Q. of unity as is given by carbohydrate (17). Acetoacetic acid, if completely oxidized, would also yield an R.Q. of 1.0. In view of the limited significance of the R.Q. of a single tissue acting on a single substrate, what possible meaning can be assigned to the composite R.Q. derived from many tissues acting on a variety of substrates? In this predicament the proponents of the R.Q. have sometimes resorted to the argument that, when the R.Q. of the whole body is determined over a sufficiently long period of time, it must represent the resultant of all the R.Q.'s in all the tissues and must therefore ultimately depend upon the chemical composition of the original substrates being oxidized. This ignores:

a. The fact that what constitutes a sufficiently long period of time, under various conditions, is difficult to determine; in any case, practical reasons have usually dictated rather short periods of R.Q. measurement in the past.

b. The possibility of partial decarboxylation of some of the intermediary metabolites of the original substrate, without further oxidation of the residues, so that the integral of the individual R.Q.'s could never equal the theoretical R.Q. of the original substrate.

c. The possibility that some oxygen is used in the formation of storage or excretion products, without the formation of equivalent amounts of carbon dioxide, with the same result as in *b*.

But even if the possible validity of the argument be granted, it is perfectly clear that the composite R.Q. cannot be used to judge the intermediate steps undergone by a substrate, on its way to complete degradation to carbon dioxide and water. In other words, even if we suppose that the R.Q. of 0.7 in diabetes means that the animal is living at the ultimate expense of fat, there is no reason for the further supposition that the fat is being directly and completely oxidized in the extra-hepatic tissues. Thus the R.Q. has no weight against the direct chemical evidence that, in its utilization, fat is converted to hexose and ketones by the liver, and that these intermediates are oxidized by the extra-hepatic tissues. It must be concluded that the determination of the utilization of a foodstuff in the liverless animal by means of chemical balance studies, however incomplete, is a far safer procedure than the attempted calculation of so-called "oxidation" from the respiratory quotient of the intact animal.

15. Attempts to Determine Carbohydrate Utilization in Intact Animals. The foregoing conclusion and our previous considerations of the

liver and extra-hepatic tissues as factors in a dynamic balance, are pertinent to the evaluation of certain attempts to determine the utilization of carbohydrate in intact animals. Wierzuchowski (279) used R.Q. measurements to calculate the amounts of sugar oxidized before and after the administration of insulin in 2 normal unanesthetized dogs receiving constant intravenous injections of glucose. According to these calculations, one of the animals oxidized 21.5 per cent of the assimilated sugar before insulin administration, and 27.3 per cent after insulin. But the other animal oxidized 19.1 per cent before insulin, and 19.0 per cent after insulin! The results from the 2 dogs were averaged to arrive at the conclusion that insulin had increased the oxidation of assimilated glucose from 20.3 per cent to 23.2 per cent.

Bissinger and Lesser (13) attempted to compare the disappearance of carbohydrate as determined by chemical analysis with the amounts of carbohydrate oxidized as computed from respiratory data. They used large groups of normal, intact mice, some of which were given intraperitoneal injections of glucose while others received glucose plus insulin. The whole bodies of control, untreated animals, and of the injected animals were minced to determine their initial and final carbohydrate contents, before and after the experimental periods. According to the respiratory data, no glucose had been oxidized up to 30 minutes after glucose alone was administered, while the animals which received glucose plus insulin oxidized 128 mgm. per cent within 30 minutes, and 159 mgm. per cent within 40 minutes. These figures for the insulin-treated animals accounted for 90 to 103 per cent of the carbohydrate which had disappeared by chemical analysis. But, in the animals which received glucose alone, 67 mgm. per cent of carbohydrate (about half the amount which disappeared in the insulin-treated animals) disappeared by chemical analysis during the time that the respiratory calculations indicated that no sugar was oxidized! In accepting these results Cori (42) brushes aside the huge discrepancy with the speculative remark that "It would be of considerable interest to know in what form the sugar is temporarily held in the tissues until it is oxidized to CO_2 and water." It might also be asked why a large proportion of the tissue carbohydrate was not also held in the same imaginary form in the mice which had received glucose plus insulin.

The latter author has himself attempted to compare chemical and respiratory computations of carbohydrate utilization in intact rats. The results are summarized in tables on pages 236 and 238 of his Review (42). It is calculated that in normal and adrenalectomized

rats, during 4 hours of glucose absorption from the gastro-intestinal tract, insulin-treated rats oxidized 5 to 12 per cent more of the absorbed glucose than did the untreated animals. This effect of insulin, however, is well within the manifest error of the experiments, for only 85 to 90 per cent of the absorbed glucose could be accounted for by the sum of oxidation, glycogen deposition, and retention in the tissues. In rats given insulin or epinephrin during the post-absorptive state, no true chemical balance was done. Respiratory data were used to calculate the carbohydrate content of the rats at the beginning of the post-absorptive period, while glycogen determinations were performed at the end of the experiment. On this basis it is calculated that insulin doubled the rate of sugar oxidation during the 3-hour post-absorptive period. But it is important to note that, on the same basis, epinephrin also increased carbohydrate oxidation by about 20 per cent. This is significant as to the reliability of the respiratory methods for, in the same Review (p. 188 et seq.), Cori discusses different respiratory work by himself and others which purports to show that epinephrin inhibits the oxidation of carbohydrate! It is apparent that the results obtained in intact animals, using the R.Q. and ignoring the formation of sugar by the liver, merely serve to confirm our previous criticisms of this method of approach.

16. Utilization of Carbohydrate as Determined by the Disappearance of the Blood Sugar in Liverless Animals. The rapid disappearance of the blood sugar after removal of the liver from the normal animal has been discussed in the previous section dealing with the site of formation of the blood sugar. The mere withdrawal of sugar from the blood by the extra-hepatic tissues cannot, of course, be regarded as proof of its utilization by those tissues. It is necessary to show that the carbohydrate does not accumulate in the tissues, and that it is not returned to the blood in altered form, *e.g.*, as lactic acid. However, it has been the universal experience that the carbohydrate content of the tissues and the accumulation of lactic acid or any other substance in the blood does not account for the sugar which disappears from the blood of the liverless animal. The rate of disappearance of blood sugar in such animals may therefore be taken as, at least, a rough indication of the utilization of sugar by the extra-hepatic tissues. In view of this, it is significant that the blood sugar disappears after hepatectomy or abdominal evisceration in animals which have been supposed to have ceased utilizing carbohydrate, as judged by the D:N, ketosis and R.Q. exhibited before removal of the liver. Such evidence is available after hepatectomy of depancreatized birds (139), dogs (178) and rabbits (88a) and after

evisceration of phlorhidzinized dogs (61) and of normal dogs fasted to the point of so-called "hunger diabetes" (255). A similar incongruity between the conclusions drawn from the classic metabolic criteria and the disappearance of the blood sugar occurs after hypophysectomy of the depancreatized dog (116) (256) and during prolonged injections of epinephrin in the normal dog (245) (259).

17. Utilization of Carbohydrate as Determined by Chemical Balance Studies in Liverless Animals. The groundwork for future chemical balance studies of carbohydrate utilization was laid in the laboratory of H. H. Dale. At that time, practical methods for total abdominal evisceration in the cat were not available. The liver was left in situ with its afferent blood supply tied off. However, the asphyxiated organ with a high free sugar content could still contribute sugar to the blood by seepage into the vena cava. In their later experiments, Dale and co-workers recognized this source of error, and corrected for it by including the changes in sugar content of the liver in their chemical balances. In these experiments, eviscerated spinal cats were given constant intravenous infusions of known amounts of dextrose. The balance was constructed from the amounts of sugar which disappeared from the blood; and from the difference in glycogen and free sugar content between certain muscles removed at the beginning of the experiment, and the corresponding muscles of the opposite leg removed at the end of the experiment. Burn and Dale (30) showed that the increase in oxygen consumption following the administration of insulin to such animals, was usually insufficient to account for more than a small part of the sugar which disappeared from the blood. Best, Hoet and Marks (12) then demonstrated that, when ample sugar was available, a large proportion of the sugar which disappeared from the blood under the influence of insulin was deposited as muscle glycogen.

Finally, Best, Dale, Hoet and Marks (11) measured oxygen consumption and performed the chemical balance on the same animals. They found that, at both high and low blood sugar levels, the sugar which disappeared from the blood during insulin action was equal to the sum of the glycogen deposited in the muscles and the glucose equivalent of the oxygen consumed. These results demonstrated two important facts as regards the validity of chemical balance studies in liverless animals:

1. There is no basis for hypothesizing an unknown intermediate substance retained in the tissues, such as Cori inferred from the work of Bissinger and Lesser.

2. Since the algebraic sum of the carbohydrate which disappears

from the blood and muscles equals the glucose-equivalent of the oxygen consumed, this sum itself can be used as a measure of carbohydrate oxidation.

In accordance with the state of knowledge at that time, Best et al. (11) concluded that the effects of insulin in excess represent an intensification of its physiological effects, including the acceleration of the combustion of carbohydrate. Hence their work has since been quoted as proof that insulin increases the oxidation of carbohydrate. Examination of their original data shows that this is not so. Table 4 summarizes the pertinent figures from the experiments which they themselves selected as being most free from technical criticism. The right hand column is our own recalculation of the amounts of sugar oxidized in milligrams per kilogram per hour, in order to make these values

TABLE 4

Influence of insulin on glucose oxidation of eviscerated spinal cats (Best, Dale, Hoet and Marks (11))

EXPT. NUMBER	ORIGINAL DATA				RECALCULATION
	Insulin	Weight of cat	Duration of experiment	Glucose oxidized	Glucose oxidized
	units	*kgm.*	*minutes*	*mgm.*	*mgm./kgm./hr.*
5A	0	3.2	50	1045	392
5B	20	3.2	150	2970	371
6	30	2.6	210	2595	285
7	25	2.8	250	3079	264

comparable. It may be seen that animal 5 oxidized less sugar after insulin than before. Animals 6 and 7, for which no pre-insulin periods are given, oxidized less sugar after insulin than animal 5 oxidized without insulin.

More recently, Soskin and co-workers have studied the utilization of carbohydrate in totally abdominally eviscerated dogs, by striking a chemical balance from the blood sugar, the blood lactic acid and the muscle glycogen at the beginning of the experiment; the amount of sugar administered in order to maintain the blood sugar at a particular level during the experiment; and the blood sugar, blood lactic acid and muscle glycogen at the end of the experiment.[3] It was found that,

[3] Some of these experiments have since been checked by determining the total carbohydrate content of the muscle instead of muscle glycogen, and lactic acid content of the muscle as well as blood lactic acid. The results were substantially the same by both methods (253a).

between certain limits, the rate of sugar utilization by the peripheral tissues of the normal dog varies directly with the height of the blood

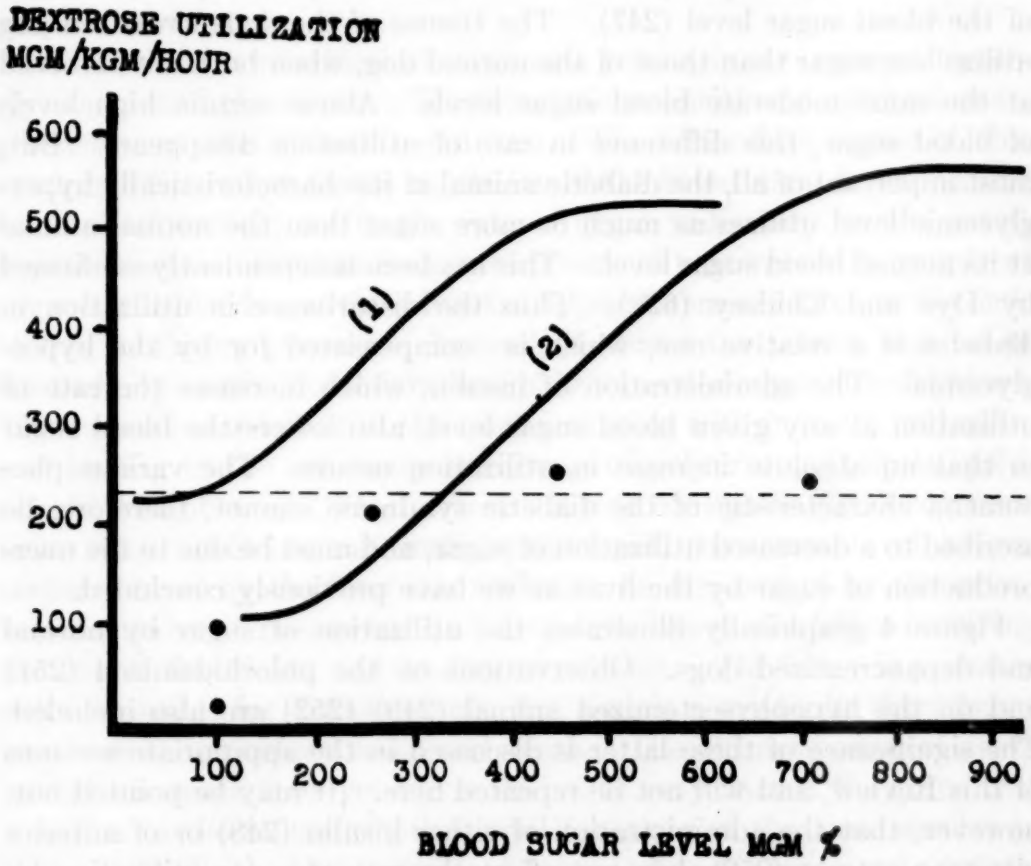


Fig. 4. The relation between the blood sugar level and the rate of sugar utilization, calculated from carbohydrate balance experiments in abdominally eviscerated dogs. The heavy continuous lines are smooth curves drawn through the scatter of individual experimental results for each type of animal. Curve *1* represents the results obtained in the normal dog (247), and in the normal dog treated with insulin (248), anterior pituitary extract (252) or phlorhidzin (251). Curve *2* represents the results obtained in depancreatized dogs (247). The dots represent individual experiments in hypophysectomized dogs (249) (252). The horizontal broken line marks the rate of sugar utilization of normal animals at the normal blood sugar level. This line crosses the diabetic and hypophysectomy curves at a level somewhat over 300 mgm. per cent. Therefore, the uncontrolled diabetic animal, with blood sugar values at or above this figure, utilizes as much sugar as the normal animal at its normal blood sugar level. But the hypophysectomized animal, which ordinarily exists at a subnormal blood sugar level, utilizes much less sugar than the normal animal.

sugar level (247). Within the normal blood sugar range of 60 to 80 mgm. per cent the normal dog utilizes about 240 mgm. per kgm. per hour, which agrees surprisingly well with the fact that Mann (177)

found it necessary to administer about ¼ gram of dextrose per kilogram per hour in order to maintain his hepatectomized dogs. Sugar utilization in the completely depancreatized dog also depends upon the height of the blood sugar level (247). The tissues of the depancreatized dog utilize less sugar than those of the normal dog, when both are observed at the same moderate blood sugar levels. Above certain high levels of blood sugar, this difference in rate of utilization disappears. But, most important of all, the diabetic animal at its characteristically hyperglycemic level utilizes as much or more sugar than the normal animal at its normal blood sugar level. This has been independently confirmed by Dye and Chidsey (62a). Thus the disturbance in utilization in diabetes is a relative one, which is compensated for by the hyperglycemia. The administration of insulin, which increases the rate of utilization at any given blood sugar level, also lowers the blood sugar so that no absolute increase in utilization occurs. The various phenomena characteristic of the diabetic syndrome cannot, therefore, be ascribed to a decreased utilization of sugar, and must be due to the overproduction of sugar by the liver as we have previously concluded.

Figure 4 graphically illustrates the utilization of sugar by normal and depancreatized dogs. Observations on the phlorhidzinized (251) and on the hypophysectomized animal (249) (252) are also included. The significance of these latter is discussed in the appropriate sections of this Review, and will not be repeated here. It may be pointed out, however, that the administration of either insulin (248) or of anterior pituitary extract (252) does not affect the rate of sugar utilization by the tissues of the *normal* dog. It may be concluded that the administration of these hormones to normal animals does not change the rate of sugar utilization, because the pancreas and hypophysis are already supplying optimal amounts of their internal secretions.

SUMMARY

18. The Mode of Action of Insulin. By summarizing the various effects of the lack or excess of insulin which have been discussed separately, it is now possible to attempt to picture its influence as a whole. To arrive at a unified conception, it will be necessary to interpolate some unproven assumptions. References are given only for those experimental findings not previously mentioned, but the difference between fact and fancy will be readily distinguishable. It is understood, of course, that one cannot really consider insulin action by itself, for it is but one factor in a balanced endocrine system. Nevertheless, it

is a useful simplification to think of carbohydrate metabolism in terms of insulin, because of the leading rôle which it has played in the historical development of the experimental and clinical aspects of the subject.

An absolute or relative lack of insulin results in a persistent hyperglycemia, with glycosuria. These are the essential and minimal characteristics of the diabetic syndrome. The immediate cause of this syndrome is a failure in the regulation of the blood sugar level. There is no absolute decrease in the rate of sugar utilization by the extrahepatic tissues. But the liver, which normally decreases its supply of sugar to the blood upon the advent of hyperglycemia, no longer responds to this inhibitory stimulus. Instead, it wastes the protein and fat of the body by continuing to form and pour unneeded sugar into the blood. The excess sugar is lost in the urine, carrying with it water and salts, and leading to the secondary symptoms of polyuria, polydipsia, polyphagia, loss of weight, etc. When the rate of hepatic gluconeogenesis from fatty acids becomes sufficiently rapid, the amounts of ketone bodies formed by the liver can no longer be disposed of by the extrahepatic tissues. Ketonemia and ketonuria ensue, the latter involving the loss of fixed base, and leading to acidosis. The latter is probably not responsible for diabetic coma, but signals a metabolic disturbance so severe as to portend coma and death. The ultimate cause of death in diabetic coma is as yet unknown. One may hazard the guess that it is due to the failure of other vital functions of the liver, similar to that which kills the hepatectomized animal when the continued administration of sugar is no longer able to support it.

To change from the negative to the positive definition of its action, insulin is an important factor in the endocrine balance which determines the normal blood sugar level. But it is not essential to the intrinsic hepatic regulating mechanism, for the latter can function to some degree in the complete absence of insulin, providing the opposing endocrine factors are also removed. By the same token, the control of clinical diabetes mellitus by administered insulin does not necessarily mean that this condition is due to a pancreatic deficiency. The introduction of extra insulin into the body can restore the endocrine balance, whether the disturbance be due to a lack of endogenous insulin or to an excess of opposing factors. It is thus possible, in view of the fact that pancreatic pathology is not usually found in diabetes mellitus, that this syndrome may include cases of pancreatic and of pituitary diabetes and, less probably, thyroid and adrenal cortical diabetes. It is even

more likely that certain cases may be termed hepatic diabetes, when the damaged liver fails to respond normally to the presence of a normal amount of insulin.

A consideration of how insulin or the lack of it produces the above effects logically seems to begin with the most firmly established effect of the hormone, namely, the withdrawal of sugar from the blood and its deposition as glycogen in the muscles. This effect can also explain the action of insulin on the liver, for when it is remembered that the liver normally puts out sugar, it will be realized that a decrease in hepatic glycogenolysis is equivalent to an increase in the rate of deposition of muscle glycogen. If it be assumed that the inhibition of glycogenolysis is the primary action of insulin in hepatic regulation, decreased gluconeogenesis from non-carbohydrate precursors may be regarded as a secondary effect. In other words, when glycogenolysis ceases while gluconeogenesis continues, there is a piling up of glycogen in the liver cells to a level which eventually stops further carbohydrate formation. This may occur through the mechanism adopted by Mirsky et al. (199) as the explanation for the antiketogenic effect of liver glycogen, namely, the predominance of the carbohydrate in the substrate competition for available oxygen (65), so that the oxidative breakdown of other foodstuffs is curtailed.

We are now in a position to explain the puzzling difference in hepatic glycogen deposition following insulin administration to the normal and diabetic organisms. When insulin inhibits glycogenolysis in the glycogen-poor diabetic liver, gluconeogenesis continues unchecked until enough glycogen accumulates to stop it. Meanwhile the excess blood sugar has been deposited in the muscles and liver so that (unless too much insulin has been given) the net result is a normal blood sugar level and increased hepatic glycogen. However, any insulin administered to the normal organism is an excess over the optimal amount already present, and causes hypoglycemia. This opposes the direct effects of insulin on the liver by bringing into operation those regulating and emergency mechanisms which result in hepatic glycogenolysis. Hence the usual finding that the glycogen stores of the normal liver are decreased after insulin administration.

It is the hypoglycemia, rather than any unknown stimulating or beneficial effect of excessive insulin on the central nervous system, which also explains the therapeutic results obtained in the insulin treatment of schizophrenia (241). It is generally agreed that nerve tissue has little stored carbohydrate and cannot utilize protein or fat. It must

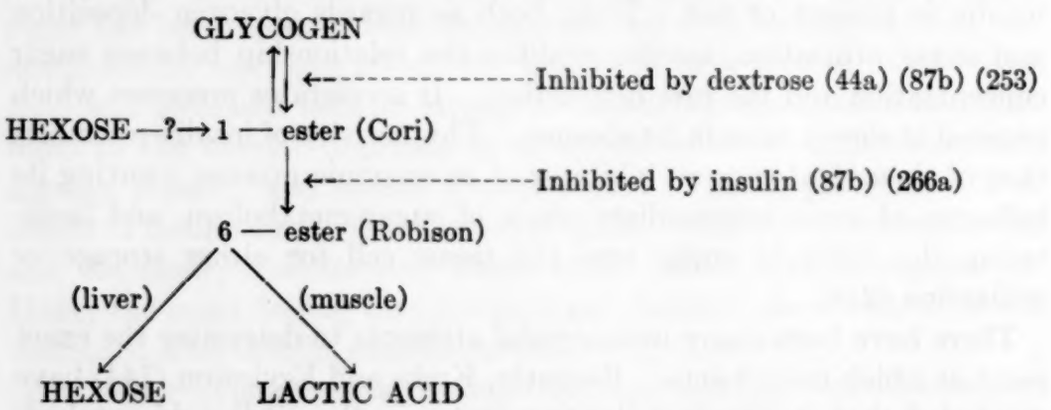
therefore depend upon the continuous supply of adequate blood sugar to maintain its vital functions. The lower plateau in the S-shaped curve which expresses the relation of the blood sugar level to the rate of sugar utilization, indicates that the latter cannot be depressed below a certain minimal rate by any degree of hypoglycemia. Marked hypoglycemia can therefore drive the supply of sugar from the blood below the amount required for the minimal rate of metabolism compatible with the well-being of the nerve tissues. Thus, insulin "shock" therapy is quite comparable to the other types of shock treatment which have been shown to yield similar therapeutic results.

The fact that the processes which are influenced by insulin have been observed to occur to some extent in the complete absence of the hormone, sheds some light on the nature of its activity. The rate of glycogen deposition depends upon the concentration of the blood sugar (44), but the presence of insulin is not essential (175) (182). Indeed, Dambrosi (49) (50) (51) and Lukens et al. (167) have demonstrated that the restoration of muscle glycogen after depletion by exercise, is just as complete in depancreatized as in normal animals, except that in the former it occurs at a much slower rate. A similar relativity holds for the influence of insulin on the utilization of sugar. In the absence of insulin the depancreatized animal can utilize carbohydrate at any rate of which the normal animal is capable. The difference between the two consists in the fact that the depancreatized animal utilizes less sugar than the normal, when the comparison is made at the same blood sugar level. Above certain high levels of blood sugar, even this difference disappears, and the same rates of utilization prevail whether insulin is present or not. Thus, both as regards glycogen deposition and sugar utilization, insulin modifies the relationship between sugar concentration and the rate of reaction. It accelerates processes which proceed at slower rates in its absence. This activity of insulin resembles that of an activator or an inhibitor of an enzymic process, exerting its influence at some intermediate phase of sugar metabolism, and facilitating the entry of sugar into the tissue cell for either storage or utilization (248).

There have been many unsuccessful attempts to determine the exact point at which insulin acts. Recently, Krebs and Eggleston (144) have concluded that insulin is a limiting factor in the citric acid catalysis of glucose oxidation, in vitro. However, the citric acid cycle has not yet been established as a recognized process in even a single type of tissue (22) (93) (127) (154). Moreover the work was done with the

minced breast muscle of pigeons, which is known to be quite resistant to the ordinary physiological effects of insulin, and which manifests little hyperglycemia or glycosuria after pancreatectomy (259d). Although Krebs and Eggleston's finding has been more or less confirmed as regards pigeon breast muscle (232a) (259c) (259d), it has not been possible to demonstrate their effect using muscles from animals which react to insulin and pancreatectomy in the usual manner. Thus, unsuccessful attempts have been made with the muscles of normal chickens, dogs, rats and cats (154) (232a) (233) (259c), and with the muscle of depancreatized cats (259c). For the time being it therefore seems proper to regard the observation of Krebs and Eggleston as an isolated phenomenon with very limited general application, occurring in an organism in which insulin does not seem to play its usual important rôle.

Another point of attack on the problem seems more hopeful. It stems from the discovery that the physiological action of insulin depends on the integrity of its S—S groups (130) (263). Although the general significance of the findings is not entirely clear, it seems probable that the sensitivity of an organism to insulin partly depends upon the level of —SH-containing substances in the tissues (150) (155). It has been shown that the formation of the Robison ester in muscle extract, which is catalyzed by the sulphydril groups, is inhibited by insulin (87b). But it has also been shown that dextrose (253) as well as insulin (266a) inhibits the appearance of free sugar in glycogenolyzing liver brei. Since dextrose is known to inhibit the formation of the Cori ester (44a) (87b), both inhibiting actions might tentatively be indicated as follows:



If this is correct, it would conform to our previous discussion regarding the primary action of insulin as an inhibitor of glycogenolysis in the liver and an activator of glycogen formation in muscle.

I am greatly indebted to Dr. R. Levine for aid with the sections dealing with intermediary metabolism.

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VITAMIN K

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Prior to 1935, several reports mentioning experimentally induced bleeding tendencies in the chicken appeared in the literature (56, 57, 71, 116, 117, 136, 137). While it is now easy to interpret these observations in terms of vitamin K, at the time of their appearance there was a lack of conclusive evidence to distinguish between several possible causes of the bleeding symptoms, such as a deficiency of an unknown dietary factor, a newly recognized effect in the chick of a deficiency of some one of the known dietary factors, or the effect of some toxic or harmful agent. Examples of bleeding tendencies and blood coagulation defects from a wide variety of causes were already known in other species.

In 1935 papers by Dam (58, 59) and by Almquist and Stokstad (24, 25) furnished strong evidence of the existence of a vitamin-like organic factor required by the chick for the maintenance of normal blood clotting power. This factor was named vitamin K by Dam. The early work on vitamin K has been the subject of several reviews (5, 10, 60, 165).

DISTRIBUTION. *Presence in plants.* Green leafy tissue is perhaps the richest natural source of the plant form of the vitamin. As little as one-half per cent of commercial dried alfalfa in the diet of the chick will meet the normal requirements (25). Dried alfalfa has been the initial source used by nearly every laboratory engaged in vitamin K isolation. The vitamin seems to be richest in the photosynthetically active parts of the plant, since the tops of carrots are a good source while the carrot roots contain no detectable quantity (6). Furthermore, the vitamin is more abundant in peas sprouted in the light than in peas sprouted in the dark (66). The inner leaves of the cabbage appear to have only one-fourth the activity of the outer leaves (66).

Among other good sources of the vitamin are spinach, cabbage and kale (66, 72), cauliflower, nettle and chestnut leaves (66). Presence of the vitamin has also been noted in the tomato, hempseed and seaweed (66), and soybean oil (27), as well as in many other minor sources.

Presence in micro-organisms. Following early observations on the

development of marked antihemorrhagic activity in foodstuffs during bacterial spoilage (24, 25) and in the droppings of vitamin K-deficient chicks (26), it was found that a number of bacteria, including the common *Bacillus cereus* and *Bacillus subtilis*, synthesize a fat-soluble anti-

TABLE 1

*Antihemorrhage activities of certain micro-organisms**

PREPARATION	COMPARATIVE POTENCY
Alfalfa standard	1
Bacillus cereus	23
Bacillus mycoides	31
Bacillus subtilis	38
Bacillus vulgatus	+
Bacterium aerogenes	4
Bacterium flexneri	6
Bacterium friedlanderi	+
Bacterium proteus	15
Bacterium typhosum	3
Chromobacterium violaceum	nil
Clostridium sporogenes	nil
Erythrobacillus prodigiosus	4
Escherichia coli	3
Myobacterium tuberculosis	11
Mycobacterium leprae	+
Pseudomonas aeruginosa	nil
Pseudomonas fluorescens	nil
Sarcina lutea	20
Staphylococcus albus	nil
Staphylococcus aureus	12
Streptococcus lacticus	nil
Aspergillus niger	nil
Monilia albicans	nil
Monilia candida	nil
Mucor, Bonar strain	nil
Neurospora cytophilia	nil
Penicillium, green	nil

* Adopted from unpublished data (23).

hemorrhagic factor even when cultured on a vitamin-K-free medium (23). Certain dried bacteria were nearly 40 times as potent as a standard dried alfalfa. Other species of bacteria did not possess measurable activity and micro-organisms of the mold, yeast and fungus types were uniformly inactive (23). Some comparative data are given in table 1.

Bacterial action on wet fish meal has been employed in the preparation of vitamin K concentrates (24, 25, 138, 175). Synthesis of the vitamin by bacteria within the intestinal tract of the chick offered an explanation for some cases of spontaneous recovery from, or resistance to, a deficient state (26). The possibility of such synthesis created a necessity for guarding against coprophagy and against bacterial action on the food of the test animals (26). The vitamin has also been found in the feces of vitamin K-deficient, bile-fistula or icteric rats (11).

Presence in tissues. In the egg the vitamin appears in the yolk, and the quantity in the newly-hatched chick is influenced by the level in the diet of the parent hen (26). The liver of young chicks (26, 65, 72) and of the rat (110) contains very little vitamin K although that of the dog and hog (72) contains moderate amounts.

The widespread occurrence of the vitamin suggests that its influence on the clotting of blood is only a part of a more general rôle.

Determination. The biological measurement of vitamin K is based upon the blood clotting power and may be conducted by curative or preventive procedure. Nearly every group of workers has devised a different assay method and has defined a different "unit" of activity, lending confusion to the general comparison of potencies. Many of the "units" had little meaning since they were based on a questionable assumption of standard response by the chick rather than on a reference standard substance. Fortunately, these "units" have been rendered obsolete by rapid progress in the identification of the vitamin and other pure compounds which serve as reproducible standards of activity.

The assay procedure of the Copenhagen group, described by Schönheyder (166) and subsequently improved by Dam and co-workers (67, 70), is so laborious that it has found little use by other workers. The vitamin K-deficient diets used have produced other conditions later traced to additional dietary deficiencies (67, 69).

The method first described by Almquist and Stokstad (27) involved preventive supplementation in the diet and comparison of whole blood clotting times of test chicks with those of positive and negative control groups. The procedure was later improved (16, 22) and based upon the "prothrombin clotting time" method of Quick (149, 152, 159). A linear relation between the reciprocal mean prothrombin clotting time and the logarithm of the vitamin K intake was found useful for the interpolation of assay results (22). More recently (17, 20) assays have been made by oral administration to deficient chicks for four days with determinations of prothrombin time on the fifth.

As compared to simple clotting time measurement, the prothrombin clotting time method was shown to produce less variable data within a test group probably through control of thromboplastin variations (16). The large variability of whole-blood clotting time in the chick is often not closely related to changes in prothrombin level (185). In the case of prolonged whole-blood clotting times there arises also a distinct possibility of deterioration of blood clotting elements during exposure of the blood. The Quick procedure has been further applied and investigated by other workers (1, 112, 140, 146, 175). A 24-hour method based on prothrombin clotting time has also been suggested (155).

The test diet of the California workers (16, 22, 27) has been used with but minor changes by most laboratories (13, 31, 32, 77, 78, 108, 155, 162, 180, 182, 184, 185, 187). This diet leads to normal growth and absence of deficiencies, other than vitamin K deficiency, within the necessary assay period. General severe deficiency occurs after 5 to 7 days when day-old chicks are placed on the diet (27, 149, 185).

A new type of diet, most of which must be heated for 1 week, has been proposed (34); this diet, however, has other deficiencies for chicks so that growth practically ceases. Multiple deficiencies should generally be avoided in biological assays. It is claimed that the diet does not permit bacterial synthesis of vitamin K within the chick or in the droppings.

Other assay methods (32, 78, 182, 187) still retain the more convenient but less accurate whole-blood clotting time. The method of Ansbacher (32) employs depleted chicks with clotting times greater than 90 minutes. Such chicks will often bleed excessively or fatally from the necessary wound, however small, within this period of observation. Severe loss of blood appears to reduce whole blood clotting time independently of vitamin K (53), but has no effect on the prothrombin time (82). The extremely short assay period of 6 hours (32) is sufficient for recovery by the chick to nearly normal blood clotting power and prothrombin level when given a sufficient dose (31, 69, 185). It seems probable, however, that such a short test would be unduly influenced by individual differences in rates of absorption and metabolism of various sources of activity. In fact, the relative activities of certain supplements have been shown to be considerably greater in an 18 hour test than in a 6 hour test (39). On the other hand, those sources of activity which develop a maximum response more rapidly may be more advantageously used in cases of clinical emergency. For a further discussion of assay methods the reader is referred to a review by Dam (61).

Physiology. It was demonstrated by the Copenhagen group (74,

166) that the impaired clotting power of deficient chick blood was due to a reduced level of prothrombin. Later reports (16, 149, 185) have fully supported this explanation. Several other species of birds, namely, the duck, goose, canary and pigeon (73), and turkey (10) are also subject to the same deficiency disease.

No other significant abnormalities have been demonstrated in vitamin K-deficient chick blood when marked hemorrhage is absent (22, 27, 28, 166). Erosions or lesions of the chick gizzard lining were included in the hemorrhagic syndrome in early reports (56, 57, 58, 116) but were later shown to be a result of a different deficiency involving biliary hypofunction (21, 24, 25).

Importance of the antihemorrhagic factor to normal mammals was difficult to demonstrate merely by removing the vitamin from the diet (73). This difficulty may have been caused by failure to deplete reserve stores in the animal, or by bacterial synthesis in the intestinal tract with some absorption (26, 110). Eventually, reports appeared of clearly identified dietary K-avitaminosis in the rat (68, 110), rabbit (63), mouse (143), and even in man (118).

Mammals in certain abnormal physiological states early provided strong evidence of vitamin K deficiency. For example, rats (112) and dogs (114) were known to exhibit loss of blood coagulability and a low prothrombin level when deprived of bile by a fistula. As a sequel to their work on the relation of bile to the absorption of the fat-soluble vitamins, Greaves and Schmidt made the observation that the low prothrombin of bile-fistula rats could be corrected by oral administration of vitamin K, but not efficiently unless the vitamin was given with bile or bile salts (112). Quick, in a résumé of the status of vitamin K, independently suggested these same relations between prothrombin, bile and vitamin K (149). Working with bile-fistula dogs and using a different quantitative procedure for prothrombin, Smith et al. (168) obtained essentially the same results as reported for the bile-fistula rat.

Ligation of the bile duct was sufficient to bring about a low prothrombin value in the blood of rats on a stock diet (112) and in chicks on a diet containing adequate vitamin K (63). It was also observed that feeding bile alone was capable of causing elevation of prothrombin level in these animals with ligated bile ducts probably through facilitated absorption of the vitamin in the diet or from bacteria in the bowel (109, 110, 112, 168). Bile alone has long been known to act against the bleeding tendencies frequently associated with human obstructive jaundice (175) but the explanation of this antihemorrhagic action has been

lacking. It is of interest to note that bile contains no appreciable amount of vitamin K (9, 110).

The bile component principally concerned in the absorption and transport of fat-soluble vitamin K from the digestive tract is undoubtedly deoxycholic acid (165). The molecular compound of vitamin K with deoxycholic acid (vitamin K-choleic acid, 11) was effective upon oral administration to bile-fistula rats (55). A crude vitamin K-deoxycholic acid preparation in aqueous solution was found effective by subcutaneous injection into deficient chicks, whereas an emulsion of the crude vitamin was effective by intramuscular but not by subcutaneous injection (69). It is pertinent to add a recent report that dioctyl sodium sulfosuccinate, a highly "surface-active" compound, can facilitate the absorption of a fat-soluble antihemorrhagic substance, methyl naphthoquinone (131).

Hemorrhagic tendencies in obstructive jaundice. From this realization of the rôle of bile and the original observations by Quick, Stanley-Brown and Bancroft of low prothrombin levels in certain cases of obstructive jaundice (159), Smith and co-workers (47, 191) and Snell et al. (175) proceeded successfully to overcome the bleeding defect in obstructive jaundice by administration of crude vitamin K with bile or bile salts. Dam and Glavind (63, 64, 65) early employed intramuscular injection of vitamin K concentrates dissolved in oil, thus dispensing with the use of bile salts which some jaundiced patients can not tolerate. Further confirmatory reports have described the clinical use of crude, purified, or synthetic sources of antihemorrhagic activity (2, 29, 49, 50, 51, 52, 127, 145, 147, 160, 161, 164, 169, 170, 176, 177, 191) administered by various routes.

Synthetic substitutes for vitamin K were found effective by intraperitoneal injection in bile-fistula rats, as absorption can proceed by this route without the agency of bile (105). Recently, the tendency has been to employ the water-soluble substitutes which can be given intravenously (2, 51, 52, 162). Highly water-soluble vitamin K substitutes now available appear to be absorbed directly from the intestine independently of bile (192).

It is generally agreed that obstructive jaundice is fully analogous, both in the fundamental nature of the bleeding tendencies and in the mechanism of the cure wrought by vitamin K and bile, to the bleeding disease of experimental animals in which drainage of bile to the intestine is prevented. The aforementioned developments bridged the gap between the dietary K-avitaminosis of the chicken and the bleeding tend-

ency of the jaundiced patient. Other clinical applications of vitamin K soon followed.

Hemorrhagic disease of the new-born. The hemorrhagic disease of the infant is now recognized as a type of alimentary vitamin K deficiency characterized by a dangerously low prothrombin level during the first few days of life (46, 123, 144, 146, 151, 156, 158). Although recovery may be spontaneous, perhaps through the establishment of an intestinal flora after ingestion of food (156, 157, 158), treatment with vitamin K orally effects a prompt recovery (75, 76, 115, 144, 148, 188, 189). Prothrombin in the blood of the infant can also be increased by giving vitamin K to the mother previous to delivery (115, 167). For a more detailed review of the hemorrhagic disease of the infant the reader is referred to papers by Quick and Grossman (156) and Grossman (113).

Hemorrhagic disease accompanying liver damage. Another type of hemorrhagic disease in which vitamin K appears to be concerned is that which sometimes follows severe liver damage. Schmidt (165) and Snell (172) called attention to liver function and liver damage as possible major factors in the metabolism of vitamin K and the synthesis of prothrombin. This idea has been given further emphasis (50, 111, 153, 154, 164, 169, 174, 190).

Trauma produced by operation and massage of the liver results in a marked loss of blood prothrombin which may last for several days (130). Extirpation of the liver also causes a striking decrease in prothrombin (30, 190) which is not corrected by the level of vitamin K with bile that suffices for the cure of biliary fistula animals (30). The hypoprothrombinemia which develops after liver injury from chronic chloroform intoxication is apparently not influenced by vitamin K (48). Cases of primary liver disease such as atrophy, hepatitis, cirrhosis and carcinoma frequently involve hypoprothrombinemia. Such cases have actually shown slow or no response to vitamin K given intravenously, intramuscularly, or orally (2, 29, 52, 147, 164). In many such cases, however, extensive liver damage apparently may exist without great loss of prothrombin, the formation of which seems to be one of the last liver functions to be impaired (50, 52, 63, 140, 173).

Hemorrhagic disease from other causes. Rarer conditions in which vitamin K absorption is hindered and prothrombin level may consequently fall include non-tropical sprue (2, 54, 84), biliary fistula (87, 170), ulcerative colitis and other abnormalities of the intestinal surface (54). Such conditions have been successfully treated with vitamin K preparations. Chicks receiving an adequate level of vitamin K in

the diet can quickly be rendered deficient in prothrombin by an addition to the diet of activated carbon, which firmly adsorbs the vitamin.[1] Feeding high levels of mineral oil in the diet causes a lowered blood prothrombin apparently by interference with absorption; this effect can be corrected by injection of vitamin K (82).

Treatment of hemorrhagic retinitis with vitamin K has yielded favorable results (141). The bleeding disease of animals fed spoiled sweet clover hay appears to resemble vitamin K deficiency in that there is a decrease in prothrombin which can be remedied by feeding good alfalfa hay (149). On this latter point there is, however, lack of agreement (171).

Hemophilia. The clotting defect in hemophilia quite clearly does not involve prothrombin deficiency (45, 50, 63, 65, 164, 159), hence it does not respond to vitamin K administration (53, 63, 164). The nature of the defect in hemophilia appears to be a lack or inavailability of blood agents which cause the change of prothrombin to thrombin, i.e., thromboplastin (45, 159).

Diagnostic tests. Diagnostic and "bedside" tests for prothrombin deficiency have been proposed most of which are based on the principle of eliminating thromboplastin variations by providing a clotting agent in excess and comparing the clotting time of the tested blood with that of normal blood under the same conditions (146, 152, 193, 194), or of comparing the thromboplastin concentrations required to produce a standard clotting time in the tested and normal blood specimens (63).

Mechanism of vitamin K action. Very little is known about the manner in which vitamin K promotes the formation of prothrombin. That the location of this action is in the liver seems to be well established.

An early suggestion that vitamin K may exist as a prosthetic group on the prothrombin molecule (74) was not supported by attempts to detect vitamin K in large quantities of the prothrombin fraction of normal chicken blood (69). Dried beef blood fed at a level of 10 per cent to deficient chicks also showed no activity.[2] It seems unlikely that the vitamin occurs in blood except during transport.

An interesting suggestion has been made that the antihemorrhagic naphthoquinones can oxidize the —SH groups of certain proteins, such as prothrombin, forming —SS— linkages between protein molecules (40). That the action of the vitamin is not simple, however, is indicated by the failure of the vitamin in contact with prothrombin deficient

[1] Unpublished data of the author.

[2] Unpublished data of the author.

chick blood to accelerate clotting (69, 74). Even the water-soluble forms, such as vitamin K-choleic acid, phthiocol, methyl naphthoquinone and the diphosphoric acid ester of methyl naphthohydroquinone, have no direct effect on deficient blood.[2]

Due to recent publicity given oxalic acid as a blood clotting agent, investigations were made of the effect of oxalic acid in vitamin K-deficient chicks and in heparinized rabbits. The oxalic acid had no detectable effect (106). Similar results were obtained in the case of vitamin K-deficient chicks after intraperitoneal injection of sodium oxalate.[2]

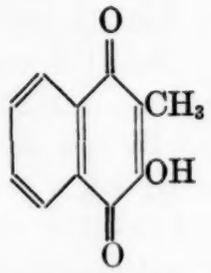
Studies of the lethal dosage of vitamin K_1 and two of its substitutes, phthiocol and methyl naphthoquinone, have indicated some toxic effects with the two substitutes at levels much above normal therapeutic levels (142). In clinical usage these compounds have given no evidence of toxic after-effects (2, 51).

The author has omitted many brief or confirmatory reports of a clinical nature. For a more complete account of the therapeutic use of vitamin K, the reader is referred to recent clinical reviews by Quick (150, 153, 154), Snell (172), Snell and Butt (173, 174), Dam (61), and Ferguson (86).

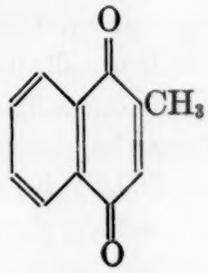
Biochemistry. Procedures which aided in the purification of the vitamin as found in alfalfa were published from time to time by Dam and co-workers (70, 72) and by Almquist and co-workers (3, 4, 6, 7, 8, 11, 125). These methods involved extraction with fat solvents, precipitation of impurities from solution in various solvents, adsorption and molecular distillation. The progress on the structure of vitamin K may be most coherently presented from the standpoint of the different groups of workers that engaged in the work.

The European group. Dam, Karrer, et al. announced the isolation of vitamin K in a highly purified form (62). The final preparation retained a constant composition and activity after repeated chromatographic adsorption. It contained carbon—82.2, hydrogen—10.7 per cent, and 2 atoms of oxygen per molecule. Absorption maxima were noted at 248, 261, 270 and 328 mμ. The extinction coefficient, $E_{1\text{ cm}}^{1\text{ per cent}}$, for the wave length 248 was 280. A characteristic color reaction of the vitamin in sodium ethylate solution was described during which an intense blue color later turning to reddish-brown developed (62, 119). Because of the fact that the product was an oil, dependable criteria for absolute purity could not be provided. Work continued in Karrer's laboratory (120) indicated a provisional formula of

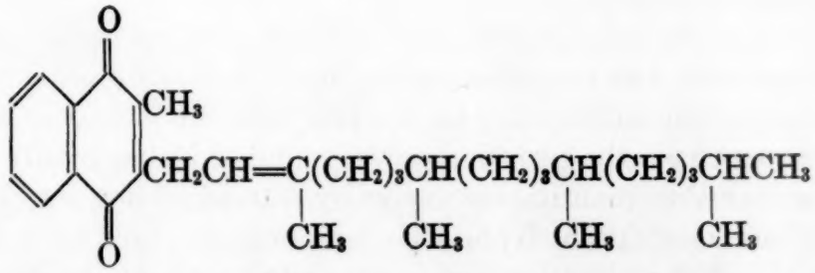
$C_{30-32}H_{46-48-50}O_2$, a molecular weight of 445–50, and that the vitamin was probably a relatively stable quinone. The name "α-phylloquinone" was suggested by Dam (121) but the information presented by the



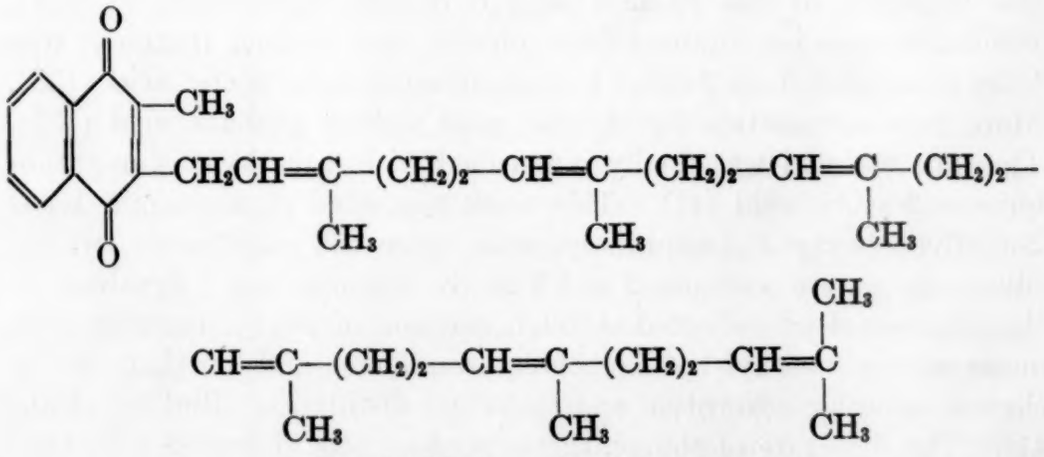
PHTHIOCOL
2-methyl-3-hydroxy-1,4-naphthoquinone



2-methyl-1,4-naphthoquinone



2-methyl-3-phytyl-1,4-naphthoquinone (vitamin K_1)



Vitamin K_2 (proposed structure)

European workers at this stage could hardly be regarded as warranting the selection of a name based on structure. Details of the final method of isolation were ultimately published (121). Synthesis of a 2-dihy-

drophytyl-1,4-naphthoquinone with physical and chemical properties similar to those of the "α-phylloquinone," except for failure of the blue color test, was also announced (122).

The St. Louis group. Independently of the European group, Doisy and co-workers at St. Louis (McKee et al., 138) had obtained two final products, one from alfalfa (K_1) and one from putrified fish meal (K_2). The K_1 extinction coefficient, $E_{1\ cm}^{1\ per\ cent}$, for the band at 248 mμ was 385, suggesting that the product was purer than the one described by Dam, Karrer et al. K_1 was a yellow oil while K_2 was a yellow crystalline solid. Nitrogen, sulphur, phosphorus and halogens were absent from both preparations. Neither was optically active. K_1 contained carbon—82.76, 82.54, hydrogen—10.65, 10.66. Its molecular weight was determined as 443, 464, and probable formula $C_{32}H_{48-50}O_2$. From physical data the suggestion was made that vitamin K contained a quinoid structure (138).

While there was some controversy over the relative purity of these preparations from the two laboratories, based primarily on the differences in extinction coefficients (42, 85, 120, 138) the biological activity of preparations from the two sources was found to be essentially identical in simultaneous quantitative assays by a third laboratory (17, 20).

Crystalline diacetates of K_1 and K_2 were next prepared by reductive acetylation and the vitamins were regenerated without change in potency (42). Ozonolysis of the K_1 diacetate led to the identification of one fragment of the vitamin as 2,6,10-trimethylpentadecanone-14, which can also be obtained from phytol, and another fragment tentatively identified as 2-ethyl-1,4-naphthoquinone-3-acetic acid (133). More drastic oxidation by chromic acid yielded phthalic acid (133). The quinone acid was finally indentified with 2-methyl-1,4-naphthoquinone-3-acetic acid (41). This work suggested that vitamin K_1 is 2-methyl-3-phytyl-1,4-naphthoquinone, since the only places left for substituents were positions 2 and 3 on the quinone ring. Synthesis of this compound was effected through reaction of phytyl bromide with monosodium-2-methyl-1,4-naphthohydroquinone and purification by chromatographic adsorption and molecular distillation (Binkley et al., 41). The diacetate of the synthetic product was identified with that of the natural vitamin K_1 (41, 135). This work was later described in more detail (134) and the procedure for isolation of the natural vitamin K_1 subsequently given (43).

The procedure for the preparation and isolation of vitamin K_2 by the St. Louis group was also described (139). In a continuation of their classical degradation studies on these vitamins, it was found that the

same quinone acid and phthalic acid could be obtained from K_2 (44). This vitamin was evidently analogous to K_1, but with a much longer and more unsaturated side chain at position 3. The correct empirical formula was given as $C_{41}H_{56}O_2$ and a structural formula was proposed (44). The ultraviolet absorption of these pure vitamins was studied in detail (85). Reviews of the work of the St. Louis group have been published (79, 80).

The Harvard group. Fieser and associates at Harvard became interested in the synthesis of vitamin K_1, after considerable information on its chemical nature had been released showing that the vitamin was probably a naphthoquinone. In a series of preliminary papers, they described steps leading to the synthesis of 2-methyl-3-phytyl-1,4-naphthoquinone which they correctly surmised to be identical with vitamin K_1 (99, 100, 101, 104). They also suggested, incorrectly, that vitamin K_2 might be 2,3-difarnesyl-1,4-naphthoquinone (95, 99). Synthesis of 2-methyl-3-phytyl-1,4-naphthoquinone was then reported by Fieser (92, 94) and similarity shown between this compound and natural vitamin K_1 in respect to absorption spectrum, color reaction, antihemorrhagic activity, melting point of the diacetate, and elementary composition (92, 93). Convenient methods for the synthesis of several of the more important antihemorrhagic compounds were reported (96). The work of this group has also been reviewed (95).

The California group. The work of Almquist and associates followed yet another course. It appeared from the effect of specific group reagents on the potency of active concentrates that the vitamin was complex, unsaturated, probably aromatic in part, and lacking in alcoholic or phenolic groups (6, 126). Reagents for ketone groups also failed to affect activity (8, 70, 126). The vitamin apparently had oily properties at room temperature and could be distilled under greatly reduced pressure (4, 8, 70). The purest concentrates contained no sulphur, phosphorus or nitrogen (6, 8). The vitamin was readily destroyed by exposure to ultraviolet light (6, 8, see also 132) and by basic substances (4, 8, 72).

Studies of the alkaline degradation products of the vitamin were pursued with the view of isolating some derivative to furnish a clue on structure. There was obtained an oily, pigmented, acidic derivative with carbon and hydrogen analyses and molecular weight suggesting a provisional formula of $C_{31}H_{50}O_4$ (18). It was apparent that this derivative was greater than the vitamin K_1 by approximately two molecules of water.

As a free acid the derived pigment was clear yellow in color, in al-

kaline solution it was reddish-brown. It became evident that the pigment was an end-stage of a color reaction for vitamin K_1 reported meanwhile by Dam, Karrer et al. (62). On the basis of the reddish-brown terminal color, close correlation between color intensity and activity was found in a variety of preparations (12).

Among a number of bacteria tested, the tuberculosis organism had been found to possess appreciable activity (23). In 1933–34, the principal pigment of this organism had been isolated and synthesized by Anderson and co-workers at Yale. The pigment, phthiocol, was 2-methyl-3-hydroxy-1,4-naphthoquinone. The attention of the California workers was drawn to this compound because it was a quinone pigment like vitamin K, because of its presence in a natural active product, because it exhibited color changes similar to those of the oily pigment derived from vitamin K by alkaline hydrolysis and because its absorption curve compared closely with that already given for preparations of natural vitamin K_1 (14, 138). A sample of synthetic phthiocol, obtained from Professor Anderson, was tested with chicks and found to be active in restoring normal blood clotting time (13, 14).

Although more potent synthetic compounds were later discovered, phthiocol is of historic importance in that it was the first pure, natural, antihemorrhagic compound of known structure to be announced and in that its activity confirmed suggestions of a quinoid character of the vitamin, indicating more specifically a naphthoquinoid structure. Phthiocol was probably the first synthetic form of vitamin K to be employed with success in human cases (51, 169).

2-methyl-1,4-naphthoquinone was found to be more active than phthiocol (14, 15) and, subsequently, by oral administration several times as active as natural vitamin K_1 (17), after low and erratic values obtained by administration of the methyl naphthoquinone in the diet were found due to some cause of loss not formerly encountered. It seemed likely that this quinone could represent the active nucleus of the vitamin and that the remainder of the vitamin not yet accounted for might logically be a phytyl side chain, because of the presence of phytol in alfalfa and because a compound of the methyl naphthoquinone and phytol could be expected to conform to the reported physical and chemical properties of vitamin K_1. Since the vitamin lacked an alcoholic group and was not split by mild alkaline hydrolysis, the phytol was evidently combined through a carbon-carbon bond as a phytyl group. Natural vitamin K_1, the alkaline derivative of the vitamin, and pure phytol, but not the methyl naphthoquinone, were also ob-

served to exhibit the same characteristic fluorescence in ultra-violet light (15). (Some synthetic samples of vitamin K_1 have not yielded this fluorescence, which may have been due entirely to free phytol present in traces.[3])

The compound 2-methyl-3-phytyl-1,4-naphthoquinone was synthesized from methyl naphthoquinone and phytol, through the hydroquinone and phytyl bromide, and purified by repeated molecular distillation and crystallization at low temperatures. The product was found to resemble natural vitamin K_1 in elementary composition, color reaction and potency (15, 124).

The first reports of the synthesis of this compound by three laboratories in this country appeared in the same issue of the same journal (15, 92, 41). It may be pointed out that the methods for synthesis did not preclude the possibility of extensive coupling at positions 5, 6, 7 and 8, although position 3 would be expected to be the most reactive. Such isomers, as well as some cyclized by-products, may well be present in synthetic preparations. It was the isolation by the St. Louis group of 2-methyl-1,4-naphthoquinone-3-acetic acid from the oxidation products of both natural and synthetic preparations which conclusively proved their identity (135).

Vitamin K substitutes. In contrast to most other vitamins, synthetic vitamin K_1 did not prove to be the ultimate goal but only a way-station. This was due to the rather exceptional fact that a portion of the vitamin was more active than the whole natural vitamin. Immediately after the report on phthiocol (13), a number of accounts appeared of activity in other naphthoquinones, mostly based on rapid 6 to 18 hour tests (35, 97, 98, 179). Ansbacher and Fernholz confirmed the activity of phthiocol and reported further that 2-methyl-1,4-naphthoquinone was practically as active as vitamin K_1 (35). This claim was supported by others (178, 187) after earlier reports of lower activity. Further assays have revealed that this compound has an even higher relative activity, between 2 and 4 times that of vitamin K_1 (17, 19, 20, 33, 78, 83, 88, 181).

Since 2-methyl-1,4-naphthoquinone can be obtained readily in a high state of purity and its physical properties are accurately known, it has been proposed as a basic standard of potency (178). It is reasonably stable to light, although the light condensation dimer is probably inactive (20). Either this compound or 2-methyl-1,4-naphthohydroquinone diacetate, which is probably more stable (85) but only one half as active (20, 37, 179), should serve very well for a standard. The di-

[3] Unpublished data of the author.

acetate appears to suffer no loss of activity, like that found with the free quinone, when given in the diet as compared to daily oral dosage.[4] The reproducibility of these compounds would add the advantage that an international standard sample need not be preserved.

2-methyl-1,4-naphthoquinone is sufficiently water-soluble at room temperature, 0.1 mgm. per ml., (0.13 mgm. per ml.[4]) to allow its use intravenously (38). One milligram daily has been suggested as an adequate clinical dose (88). The methyl naphthohydroquinone is more water-soluble but equally as active (20, 38, 178), probably because it is readily subject to oxidation on contact with air.

The compound, 4-amino-2-methyl-1-naphthol hydrochloride, is quite water-soluble and approximately as potent as vitamin K_1 (81). It has also been variously reported to be about one-half as potent (20) and equally as potent (162) as methyl naphthoquinone into which it is probably metabolized (80). 4-amino-3-methyl-1-naphthol hydrochloride is similarly active (162).

A large number of compounds capable of conversion to 2-methyl-1,4-naphthoquinone by easy stages during metabolism probably owe their activity to such conversion. Among these compounds are 1-hydroxy-2-methyl and 1-hydroxy-3-methyl naphthalene, 1-amino-2-methyl naphthalene, 3-methyl-1-tetralone, and 2-methyl-1-tetralone (186). In this same report it was shown that 1-methyl-2-hydroxy, 2-methyl-3-hydroxy, and 1-methyl-4-hydroxy naphthalenes were inactive at high levels. The latter compounds are not convertible to 2-methyl-1,4-naphthoquinone. The comparatively low potencies of compounds like 2-methyl-3-hydroxy, 2,3-dimethyl, and 2-ethyl-1,4-naphthoquinone are thus explainable on the basis that removal of the hydroxy or the second methyl group or conversion of the ethyl to a methyl group would not be an easy metabolic process.

A series of di-esters of the methyl naphthohydroquinone were prepared and found potent, probably because of hydrolytic regeneration of the methyl naphthoquinone (37). An apparent exception was encountered in the case of the quite active 2-methyl-1,4-dimethoxynaphthalene, which presumably could not be readily hydrolyzed (37), but it has been pointed out that this compound could be oxidized to the methyl naphthoquinone (186).

Comparative assays of vitamins K_1 and K_2 have indicated that the potencies of these compounds are in the ratio of 1.25 to 1, which is also the ratio of the percentage of methyl naphthoquinone combined in each

[4] Unpublished data of the author.

(20). Assays by an 18 hour procedure indicated a higher potency ratio of 1.52 (179, 182). It is obvious that the long side chains in these vitamins are not specific; in fact, the methyl naphthoquinone is more active without the side chains (17, 20) which are probably removed in metabolism.

Numerous other naphthoquinones have been found active to some degree (20, 89, 91, 97, 103, 179, 187). Following phthiocol, several naturally occurring 3-hydroxy naphthoquinones, having a longer side chain in place of the methyl group, i.e., lapachol, hydrolapachol and lomatiol, were reported to show some activity (97), but another report at the same time indicated no activity (14) and was later confirmed (99). Various derivatives of phthiocol were assayed but no exceptionally high potency found (15). The list of antihemorrhagic substances is now too large to be given in detail. Many of these compounds do not possess practical value.

The most surprising development in recent studies of simple, synthetic vitamin K substitutes is the report of Foster et al. that 2-methyl-1,4-naphthohydroquinone-diphosphoric acid ester is even more active on the molecular basis than the methyl naphthoquinone (108). This fact encouraged the suggestion that the metabolism of methyl naphthoquinone might involve phosphorylation as an early step (108), but, of course, it could also mean that the diphosphoric acid ester is more efficiently absorbed than any other antihemorrhagic compound, and that after absorption the quinone is readily regenerated. Either assumption could explain the lower activity of the disulphuric acid ester (102, 162) from which the quinone would not be so readily recovered. Another report has maintained that the activity of the diphosphoric acid ester is but 1/20 that of the methyl naphthoquinone (38). The high activity of the diphosphoric acid ester has been amply confirmed, however, and appears to be approximately 50 per cent greater on the molecular basis than that of the methyl naphthoquinone (20, 107). Pharmacological studies on this compound indicate a wide margin of safety and no adverse effects in therapeutic doses (107, 108).

Claims for an additional form of vitamin K in alfalfa have been made. These are based on the separation from alfalfa extracts of a fraction which did not give the typical strong color reaction in sodium ethylate, yet manifested considerable activity (39, 90). It was nearly colorless and was later reported to be more active than synthetic 2-methyl-3-phytyl-1,4-naphthoquinone in a 6 hour test, but not in an 18 hour test (39). 2-methyl-3-"isophytyl"-1,4-naphthoquinone was prepared

and found comparable to synthetic vitamin K_1 in activity (39). It is interesting, in this connection, that vitamin K_1 can be converted to a 2,3-oxido derivative, a nearly colorless oil, which is as active as vitamin K_1 but which gives no Dam-Karrer color reaction, and resembles in these respects the second form of vitamin K reported to be present in alfalfa (103).

The activity of these nearly colorless products (and of the colorless reduced forms and diesters of the vitamins) may explain the potency found in some earlier, semi-refined, preparations which had very little or no color (7, 8, 129, 183) and in which the vitamin may have been present in a mixed crystal, or perhaps adsorbed on an inert crystalline medium.

TABLE 2

Comparative activities of the more important antihemorrhagic compounds based on recent chick 5-day assays and expressed in 2-methyl-1,4-naphthoquinone units per milligram and per micromol

	UNITS PER MILLIGRAM	UNITS PER MICROMOL
2-methyl-1,4-naphthoquinone	1,000	172
2-methyl-1,4-naphthohydroquinone	930	162
2-methyl-1,4-naphthohydroquinone diacetate	450	116
2-methyl-4-amino-1-naphthol hydrochloride	470	99
2-methyl-1,4-naphthohydroquinone-diphosphoric acid ester (tetra sodium salt + 6 molecules water)	490	260
2-methyl-3-phytyl-1,4-naphthoquinone (vitamin K_1):		
Natural	300	136
Natural, dihydro diacetate	100	54
Synthetic	290	131
2-methyl-3-(?)-1,4-naphthoquinone (vitamin K_2)	240	139

Only one outstanding example of activity is definitely known where conversion to methyl naphthoquinone does not seem possible. This is in the case of 2,5-dimethyl-benzoquinone (36) which has on both sides of its structure the same configuration as present along the 1, 2, 3, 4 positions of 2-methyl-1,4-naphthoquinone. Antihemorrhagic activity of this benzoquinone, although very slight, was detectable and has been confirmed (approximately 1/10,000 that of methyl naphthoquinone[5]). A degradation product of vitamin E, α-tocopherylquinone, apparently has some slight antihemorrhagic activity (128). Activity comparable to that of 2,5-dimethyl-benzoquinone has also been found in perezone, which is probably a benzoquinone.[5] It seems possible,

[5] Unpublished data of the author.

therefore, that certain compounds may act feebly in their original form or through some general property of the quinones.

Vitamin K, like the other fat-soluble vitamins, has now expanded into an extensive group of biologically active compounds. It would not be surprising if additional, naturally occurring or synthetic forms should be discovered. A comparison of the activities of some of the more important known compounds is given in table 2.

The author is indebted to Professors C. L. A. Schmidt, L. W. Taylor and S. Lepkovsky and Mr. A. A. Klose who read and criticized the manuscript.

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